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Immunological Studies on Staphylococcal Superantigen-like Proteins

A thesis submitted by

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For the degree of

Doctor of Philosophy

in the University of London

2005

Department of Immunology and Molecular pathology

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ABSTRACT

The staphylococcal superantigen-like proteins (SSLs) are a family of polymorphic paralogs encoded within the *Staphylococcus aureus* genome, whose function remains unknown. The ability of SSL7, and a closely related paralog SSL9, to interact with cells of the immune system was investigated. Within the populations of human white blood cells, both SSLs interact selectively with monocytes, via specific but separate binding sites, leading to rapid uptake of SSLs. In addition, SSLs are rapidly taken up by dendritic cells (DC), but not macrophages, and target the mannose-receptor dependent endosomal antigen processing pathway. The effect of these proteins on the functional capacity of antigen presenting cells to uptake and present antigens to T cells was also determined. Neither SSL was toxic to DCs and the presence of SSL protein did not inhibit the antigen presenting cell activity, in terms of stimulation of either allogeneic or recall T cell responses. The immunological response to the SSL proteins in the normal human population was investigated. More than 30% of healthy normal subjects tested showed T cell responses to both SSL7 and SSL9. Moreover, almost all individuals had specific non-cross reacting antibodies against this family of proteins. In order to identify the SSL receptor(s), affinity chromatography techniques were used to isolate and identify the receptor(s) from selected cell lines. A single specific protein band that may represent the putative SSL receptor was observed, and mass spectrometry identified a candidate binding protein as heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa, GRP78 (BiP)). The presence of eleven members of this protein family within the pathogenicity island SaPI_n2 in almost all *S. aureus* strains tested so far, suggest that these proteins have important non-redundant biological functions as agents of host/pathogen interactions. The data presented in this thesis further suggests that this function may involve targeting the host antigen presenting cell.

DEDECATION

In memory of my father-in-law



Mr. Mohammed A. Al-Sagheir

Former deputy-Minster for financial affairs, Ministry of Information, KSA.

During the second year of my PhD, Mr. Al-Sagheir passed away, having fought a short and hard battle against hepatic cancer. His death left us all devastated.

He devoted himself to his community, loved and trusted his family and friends, always dedicated himself to doing what was right and never surrendered until his last breath.

He will be dreadfully missed by everyone who had the good fortune to enjoy his friendship.

May Almighty God reward him for all his invaluable services and rest his soul in peace. Amen

I dedicate this thesis to him.

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There are many others who, in so many ways, have contributed and assisted to this work. I express my sincere thanks to them though they are not identified. In addition, there are people who have taught me over the years and I will always be grateful to them.

Finally, I'm grateful to the Ministry of Health, Kingdom of Saudi Arabia for the financial support of my PhD project.

PUBLICATIONS

Data and concepts in this thesis have contributed to the following publications (attached at the end of the thesis):

- **Al-Shangiti, A. M.**, Nair, S. P., and Chain, B. M. The interaction between Staphylococcal superantigen-like proteins and human dendritic cells.
(**Accepted**, Clin Exp Immunol, 2005 Feb).
- **Al-Shangiti, A. M.**, Naylor, C. E., Nair, S. P., Briggs, D. C., Henderson, B., and Chain, B. M. Structural relationships and cellular tropism of staphylococcal superantigen-like proteins.
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ABBREVIATIONS

AGR	Accessory Gene Regulator
APC	Antigen Presenting Cells
APS	Ammonium Persulphate
BiP	Immunoglobulin heavy chain binding protein
B-PER	Bacterial Protein Extraction Reagent
CARD	Caspase-recruitment domains
CD	Clusters of Differentiation
CHCA	α -cyano-4-hydroxycinnamic acid matrix
CM	Complete Medium
CR	Complement Receptors
CRP	C-Reactive Protein
DCs	Dendritic Cells
DC-SIGN	DC-specific intercellular adhesion molecule (ICAM) grabbing non-integrin
DMSO	Dimethyl sulfoxide
ER	Endoplasmic Reticulum
ET	Exofolative Toxin
FCS	Fetal Calf Serum
FITC	Fluorescein isothiocyanate
GM-CSF	Granulocyte-Macrophage Colony Stimulating Factor
GRP	Glucose Regulated Protein

GST	Glutathione <i>S</i> -transferase
HBSS	Hank's Buffered Saline Solution
HSP	Heat Shock Proteins
ICAM-1	Intercellular adhesion molecule-1
IFN-γ	Interferon- γ
IgG	Immunoglobulin G
IL	Interleukin
imDC	Immature DC
INCSS	International Nomenclature Committee for Staphylococcal Superantigen
IPC	Interferon Producing Cell
IPTG	Isopropyl-B-D-thiogalactopyranside
KDa	Kilo Dalton
LBP	Lipopolysaccharide Binding Protein
LC	Langerhans Cell
LFA	Leukocyte Functional Antigen
LPS	Lipopolysaccharide
LTA	Lipoteichoic Acid
MAbs	Monoclonal Antibodies
MACRO	Macrophage Receptor with a Collagenous Structure
MALDI-TOF	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
MBL	Mannose Binding Lectin
mDC	Mature DC
MHC	Major Histocompatibility Complex

MLR	Mixed Leukocyte Reaction
MR	Mannose Receptor
Ni-NTA	Ni-nitrilotriacetic acid-agarose columns
NK	Natural Killer
NOD	Nucleotide-binding oligomerization domains
PAMPs	Pathogen Associated Molecular Patterns
PBMCs	Peripheral Blood Mononuclear Cells
PDC	Plasmacytoid DC
PG	Peptidoglycan
PMSF	Phenylmethyl-sulfonyl fluoride
PPD	Purified Protein Derivative
PRR	Pattern Recognition Receptors
RBC	Red Blood Cell
SAGs	Superantigens
SAP	Serum Amyloid Protein
SAR	Staphylococcal Accessory Regulator
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SEs	Staphylococcal Enterotoxins
SET	Staphylococcal Exotoxin Like
SR	Scavenger Receptor
SSL	Staphylococcal Superantigen Like
TCR	T Cell Receptor
TEMPD	N,N,N',N'-tetramethylethylenediamine
TFA	Tri-fluoroacetic acid

Th	T helper
TLR	Toll Like Receptor
TNF	Tumor Necrosis Factor- α
TSS	Toxic Shock Syndrome
TSST-1	Toxic Shock Syndrome Toxin-1
TT	Tetanus Toxoid
UK	United Kingdom

CHAPTER I

General Introduction

1. Introduction

1.1. Immunity to bacterial infection

1.1.1. Host/Microbial interaction

A pathogen is an organism that can overcome the defence mechanisms of the body and induce deleterious changes in the host. Protective mechanisms are a series of systems which either confine the micro-organisms to the mucosal or skin surfaces, preventing access to the tissues of the body or, if the organism evades these outer defences, then there are systems that detect, confine and eliminate the invader (Basset et al., 2003). The host–microbial relationship is a dynamic process in which the microbe attempts to minimize its visibility, to insure survival, whereas the host attempts to prevent and eradicate infection with minimal damage to self (Palucka and Banchereau, 2002).

Antimicrobial protection is ensured by the coordinated action of the innate and adaptive immune systems (Janeway, Jr. and Medzhitov, 2002). The innate immune system is composed of two elements: cells such as epithelial cells, neutrophils, natural killer (NK) cells, macrophages and dendritic cells (DCs); and, proteins such as cytokines that are produced by the cells of the immune system or proteins such as complement factors that are produced by nonimmune cells (Palucka and Banchereau, 2002).

Interaction of the host with the bacteria occurs at three levels: the extra-epithelial, epithelial and sub-epithelial level (Basset et al., 2003). The first line of defence is the epithelial surfaces of the airways and gastrointestinal tract, which face a mixture of mostly non-pathogenic bacteria from food, commensal microbes, and some pathogenic bacteria. In the airways, the majority of the microbes are removed by the overlying mucociliary escalator. Furthermore, respiratory epithelia are coated with a thin layer of secretions, which contain antimicrobial agents, for example lysozyme and defensins (Ganz, 2002). Another level of protection is provided by the tight junctions that connect the epithelial cells, forming a strong barrier that prevents the entry of microbes and their products. However, bacterial binding to the epithelium triggers a series of alarm signals resulting in the secretion of chemokines to recruit other components of the innate system, leading to the development of an acute inflammatory reaction (Basset et al., 2003). A small proportion of incoming microbes that enter at sites of micro-lesions is then handled by antigen-presenting cells (APCs), which are mostly DCs. Thus, when a pathogen invades a tissue, the immune system faces several challenges. First, it must sense a pathogen and then deliver an appropriate adaptive immune response (Palucka and Banchereau, 2002).

The adaptive immune response is associated with the activation of effector cells, B and T lymphocytes, that are capable of specific recognition of microbial antigens, and is accompanied by the generation of memory cells that prevent infection with the same pathogen in future. Together, the innate and adaptive immune systems form an integrated host defence machinery to confer optimal protection (Moll, 2003).

1.1.2. Immune recognition of bacteria:

Within the bacteria, there are four groups of especially significant human pathogens; these are Gram-negative and Gram-positive bacteria, mycobacteria and spirochaetes (Aderem and Ulevitch, 2000). A wide variety of bacterial components are capable of stimulating immune responses. After engagement of a pathogen with the epithelial surface and other components of the innate immune system such as DCs and macrophages, signals are generated that result in the production of chemokines and cytokines (Basset et al., 2003; Philpott et al., 2001). The basic feature of this activation of the innate immune system is pattern recognition (Janeway, Jr. and Medzhitov, 2002).

Pathogens are identified by the immune system by specific arrangements of key molecules called pathogen-associated molecular patterns (PAMPs) which are recognised by pattern recognition receptors (PRRs) (Table 1). The PAMPs are vital structures of the microbial cell such as lipopolysaccharide (LPS), peptidoglycan (PG), and oligosaccharides. The PRRs are found on many cells of the innate immune system (cell-associated receptors) including epithelial cells, macrophage-monocytes, granulocytes, mast cells and DCs. There are several different families of PRRs such as Toll-like receptors (TLR), scavenger receptors, mannose receptors, complement receptors (CR3) and CD14. There are also several soluble receptors, which bind PAMPs, such as CD14 (also found associated with cells), lipopolysaccharide-binding protein (LBP) and the mannose-binding lectin (MBL) (Basset et al., 2003; Janeway, Jr. and Medzhitov, 2002). The main functions of PRR include opsonization, activation of complement cascades, phagocytosis, activation of proinflammatory signaling pathways, and induction of apoptosis (Janeway, Jr. and Medzhitov, 2002).

Table 1-1: Bacterial PAMPs and pattern recognition receptors

PAMP	Pathogen(s)	PRR
LPS	Most Gram negative bacteria	LBP, CD14 TLR4, TLR2 Scavenger receptor
Lipoproteins	Eubacteria	TLR2
Peptidoglycan	Most Gram positive bacteria	TLR2 NOD1/2
Lipotechoic acid	Many Gram positive bacteria	TLR2
Flagellin	Bacteria	TLR5
Mannans and mannoproteins	Yeast	Mannose receptor Mannose-binding protein
CpG DNA	Bacteria	TLR9
Lipoarabinomannan	Mycobacteria	TLR2 CD1

(Aderem and Ulevitch, 2000; Janeway, Jr. and Medzhitov, 2002; Philpott and Girardin, 2004)

1.1.3. Receptors involved in bacterial recognition

1.1.3.1. *Toll-like receptors (TLR)*

The Toll-like receptors are a set of conserved proteins that have been found in plants, insects and mammals (Underhill and Ozinsky, 2002). In humans, there are 10 TLRs with differing ligand-binding specificities (Takeda et al., 2003). TLRs are found on a wide range of cells including immune cells such as mast cells, macrophages and DCs. TLR2 interacts with peptidoglycan, lipopeptides and other products from Gram-positive bacteria, acid fast bacteria and fungi, whereas TLR4 recognizes LPS produced by most Gram-negative bacteria (Takeda et al., 2003). Further diversification in microbial recognition is provided by TLR5, which recognizes bacterial flagellin (Hayashi et al., 2001), and by TLR3, which recognizes double-stranded RNA produced during the course of many viral infections (Alexopoulou et al., 2001). Signaling pathways used by TLRs universally lead to activation of the transcription factor NF- κ B and mitogen-activation protein kinases, general production of cytokines such as interleukin (IL)-6, IL-10 and IL-12, and expression of co-stimulatory molecules including CD40 and CD86 (Takeda et al., 2003).

1.1.3.2. *Scavenger receptors*

Scavenger receptors (SR) (e.g. SR-AI, SR-AII and MARCO [MØ receptor with a collagenous structure]) are expressed by myeloid cells (macrophages and DCs) and certain endothelial cells (Peiser et al., 2002). They bind and internalise bacteria and their products, including Gram-positive bacteria (lipoteichoic acid), Gram-negative bacteria (LPS), and are involved in clearing tissue sites of invading organisms by acting as receptors for phagocytosis (Basset et al., 2003).

1.1.3.3. *The mannose receptor family*

The mannose receptor family is a subgroup of the C-type lectin superfamily (Weis et al., 1998) and comprises four members; the mannose receptor (MR), the M-type phospholipase A₂ receptor (PLA₂R), DEC-205 and Endo180. The C-type lectin superfamily is a large group of transmembrane receptor and soluble proteins including the selectins, asialoglycoprotein receptor and collectins (East and Isacke, 2002). A characteristic feature of this family is that they all have the ability to be rapidly internalised from the plasma membrane via clathrin-coated vesicles for delivery into the endosomal system. MR receptor has been demonstrated to bind a wide variety of bacteria such as *Mycobacterium tuberculosis* and *Klebsiella pneumoniae* (East and Isacke, 2002).

DC-specific intercellular adhesion molecule (ICAM) grabbing non-integrin (DC-SIGN; CD209) is another type of C-type lectin specifically expressed by DCs and has two functions. Firstly, as an adhesion receptor, DC-SIGN supports initial DC/T cell interaction by binding to ICAM-3 (Geijtenbeek et al., 2000). Secondly, as a pathogen-recognition receptor, DC-SIGN was also shown to bind microorganisms such as *Leishmania* (Colmenares et al., 2002), and *Mycobacterium* (Geijtenbeek et al., 2003).

1.1.3.4. Soluble receptors

Mannan-binding lectin (MBL), C-reactive protein (CRP), and serum amyloid protein (SAP) are secreted pattern recognition molecules produced by the liver during the acute phase response at the early stages of infection (Schwalbe et al., 1992; Gewurz et al., 1982). CRP and SAP are members of the pentraxin family, and both can function as opsonins upon binding to phosphorylcholine on bacterial surfaces (Schwalbe et al., 1992; Gewurz et al., 1982). CRP and SAP can also bind to C1q and thus activate the classical complement pathway (Agrawal et al., 2001). MBL is a member of the collectin family that binds specifically to terminal mannose residues, which are abundant on the surface of many microorganisms (Janeway, Jr. and Medzhitov, 2002).

Another group of proteins involved in intercellular pattern recognition is the family of NOD (nucleotide-binding oligomerization domains) proteins. NOD1 and NOD2 have one and two caspase-recruitment domains (CARDs), respectively, as N-terminal effector domains (Philpott and Girardin, 2004). Both NODs acts as a bacterial peptidoglycan recognition molecule through specific detection of the conserved muramyl dipeptide structure (Girardin et al., 2003; Philpott and Girardin, 2004).

1.2. Role of dendritic cells in bacterial infection

1.2.1. DC physiology

Secondary lymphoid organs provide the specialized microenvironment required for generating a specific immune response. Initiating immunity to a microbe that invades peripheral tissues thus requires linking of the infected peripheral site with secondary lymphoid organs. The dendritic cell is a crucial link between these two sites (Wick, 2002). They exist in distinct stages depending on their location and signals in their immediate environment, and have the capacity to migrate. The DC is also the most efficient type of APC that stimulates naïve T cells. It is these unique properties of DCs that give them a central role in initiating and modulating immune responses (Banchereau and Steinman, 1998).

DCs are produced from haematopoietic stem cells within the bone marrow and can be divided into subsets according to surface marker expression, specialized functions and tissue distribution (Liu, 2001). Immature DCs are found in blood, lymphoid and non-lymphoid tissues where they perform a sentinel function for incoming pathogen, whereas mature DCs are primarily found in lymphoid tissues. In humans, two types of DC have been described: myeloid ($CD11c^{+}$) and plasmacytoid ($CD11c^{-}$) DCs. Myeloid DCs include epidermal DCs [also called Langerhans cells (LCs)] and dermal or interstitial DCs, which can also be found in other peripheral tissues (Liu, 2001). Additionally, immature myeloid DCs can be found in circulating blood or can be generated from blood precursors during their transit in peripheral tissues; these precursors are monocytes and the precursors of plasmacytoid DCs (PDCs) (Liu, 2001). PDCs are also known as type I

interferon-producing cells (IPCs) owing to their capacity to release type I interferons after viral challenge (Colonna et al., 2002).

Monocytes (CD14⁺) can differentiate into cells displaying features of immature DC or macrophages in response to GM-CSF and IL-4 or M-CSF, respectively (Sallusto and Lanzavecchia, 1994). However, monocyte differentiation is reversible and immature monocyte-derived DC or macrophages can interconvert into one another until late stages of their differentiation/maturation process (Palucka et al., 1998).

1.2.2. Interaction of DCs with bacteria

1.2.2.1. DCs as microbial sensors

When a microbe infects a tissue, resident immature DCs sense the microbe by recognizing evolutionarily conserved molecular patterns that are integral to microbial carbohydrates, lipids, and nucleic acids (or PAMPs). This is achieved through PRRs such as TLRs and MRs (Janeway, Jr. and Medzhitov, 2002). Mammalian TLRs have broad specificity for conserved molecular patterns shared by large groups of pathogens [such as LPS in Gram-negative bacteria and bacterial CpG DNA]. It appears that TLRs offer DCs a means of discriminating between different bacterial stimuli. Thus, *Escherichia coli* LPS signals through TLR4; peptidoglycans from *Staphylococcus aureus* and zymosan signal through TLR2; CpG bacterial DNA signals through TLR9; and bacterial flagellin signals through TLR5 (Alexopoulou et al., 2001; Hayashi et al., 2001; Takeda et al., 2003).

1.2.2.2. Activation of DCs by microbial products

Once a DC has detected a specific microbe, information about the pathogen is then relayed to naïve T lymphocytes in the draining lymph nodes, in a sequence of events. First, immature DCs (imDCs) capture the microbe or its products by several mechanisms, including the actin-dependent process of phagocytosis (for particulate antigens) and receptor-mediated endocytosis or macropinocytosis (for soluble antigens) (Banchereau and Steinman, 1998). Then immature DCs exit the site of infection and migrate toward the T cell areas of the proximal lymph nodes via afferent lymphatics. During this journey, DCs differentiate into mature DCs (mDCs), losing their antigen-capturing capacities but acquiring the capacity to process and display peptide antigens on their surface, in conjunction with molecules of the major histocompatibility complex (MHC) (Table 2) (Banchereau and Steinman, 1998). To generate CD8⁺ cytotoxic killer cells, DC have to present antigenic peptides in the context of MHC class I molecules (Watts, 1997). Besides the classical presentation of processed antigen in the context of MHC class I and class II, DC can also employ the CD1 (hallmark of the DC phenotype) pathway to present microbial lipid containing antigen and induce an immune response to infectious agents, particularly *Mycobacteria* (Palucka and Banchereau, 1999).

For productive immunity to occur, DCs must present not only peptide-MHC complexes but also additional costimulatory signals (such as molecules CD80 and CD86) to T cells. The interaction between CD86 and its corresponding ligand CD28 on T cells results in the up-regulation of CD40 ligand on T cells. The T cells may then engage CD40 on DCs and trigger a burst of cytokine expression, including IL-12, which induces IFN- γ in T cells (Banchereau and Steinman, 1998). Signaling through CD40 also up-regulates numerous other costimulatory molecules, which may play distinctive roles in tuning the immune response (Banchereau and Steinman, 1998; Pulendran et al., 2001).

Table 1-2: Activation of DC function by microbial stimuli

Migration

Exit of active DCs from peripheral tissues

Entry into T cell areas of secondary lymphoid tissues

Antigen presentation

Antigen presenting molecule upregulation (MHCI, II and CD1)

Formation of MHCII and peptides complexes

Upregulation of T cell interaction molecules

Costimulatory molecules [CD80, CD86]

Adhesion molecules (ICAM-1)

Signaling molecules (CD40)

Cytokine production

Induction of IL-2, TNF, IL-10, IL-6, IFN- α/β

(Reis e Sousa et al., 1999)

1.2.2.3. Antigen presentation and T cell activation

The ability to prime naive T cells constitutes a unique and critical function of DC. Following antigen uptake DC migrate to the lymph nodes and, meanwhile, mature from Ag-capturing cells to become Ag-presenting/activating cells (Banchereau and Steinman, 1998). *In vitro*, DCs induce the mixed leukocyte reaction (MLR), where only one DC is necessary to activate 100-3000 T cells. The high efficiency of T cell activation may be due to the persistent expression of high levels of antigens and costimulatory molecules. For example, MHC-peptide complexes are 10-100 times higher on DC than on other APC like B cells and monocytes. Recognition of MHC/peptide complexes on DC by antigen-specific T cell receptor (TCR) constitutes the "first signal" in DC-T cell interaction (Banchereau and Steinman, 1998). This initial step is strengthened by high expression of several adhesion molecules, like integrins B1 and B2 and members of the immunoglobulin superfamily (CD2, CD50/ICAM-3, CD54/ICAM-1, and CD58/LFA-3). Several accessory molecules, expressed on DC (CD80, CD86, CD40), interact with ligands and counter-receptors on T cells, constituting the "second signal", which is required to sustain T cell activation (Palucka and Banchereau, 1999; Banchereau and Steinman, 1998).

Activated CD4⁺ T lymphocytes can differentiate into either T helper 1 (Th1) or T helper 2 (Th2) cells that secrete specific types of cytokines. In general, Th1 cells secrete the cytokines IFN- γ and TNF- α , and are associated with a strong cell-mediated response, whereas Th2 lymphocytes secrete the cytokines IL-4, IL-5, IL-10, and IL-13, and are characterized by a humoral or antibody-mediated immune response (Lucey et al., 1996).

The activation of the Th1 immune response is dependent on IL-12, which is secreted by macrophages and DC (O'Garra et al., 1995).

1.2.3. Bacterial evasion of DC function

Infectious agents evolved and established a multitude of strategies to interfere with the development of host immunity at several levels (Moll, 2003). As dendritic cells have a key function in initiating and connecting both arms of the immune responses, the suppression of dendritic cell maturation, migration and antigen presentation as well as the inhibition of dendritic cell-mediated T cell activation provide means to facilitate the survival of microbial pathogens inside the hosts (Palucka and Banchereau, 2002; Moll, 2003). Several recent studies have documented the modulation or impairment of DC functions by bacteria including *Salmonella*, *Mycobacteria*, *Streptococcus*, *Neisseria* and *Helicobacter*, or bacterial toxins such as anthrax lethal and *E. coli* heat labile toxins.

1.2.3.1. Interference with DC generation and survival

Pathogens can affect DC migration to the infected tissues, their differentiation and their survival (Palucka and Banchereau, 2002). Induction of DC apoptosis by phagocytosed bacteria may be regarded as a microbial strategy to evade the immune system. However, this mechanism could be an effective host action to limit intracellular bacterial replication (Colino and Snapper, 2003). The ability of *Helicobacter pylori* to induce apoptosis in human monocytes suggests that this may be an important strategy for the evasion of immune recognition through a reduction in the number of imDC (Galgani et al., 2004). Furthermore, bacteria can affect DC viability, in particular, *Salmonella typhimurium* (van

der Velden et al., 2003) and *Streptococcus pneumoniae* (Colino and Snapper, 2003) can rapidly kill DC by induction of apoptosis.

1.2.3.2. Interference with antigen-presentation functions

Microbes have developed several means to interfere with the ability of DCs to present antigens to T cells preventing the induction of specific immune responses. For instance, microbes can prevent DC maturation either directly or indirectly and this inhibition has two beneficial consequences for microbes, on the one hand, prevention of microbe-specific immunity and on the other, induction of microbe-specific tolerance, when immature DCs present microbial antigens in the absence of costimulatory signals (Palucka and Banchereau, 2002).

Kikuchi et al (2004) demonstrated that the Gram-negative bacillus, *Legionella pneumophila*, has the ability to impair DCs maturation (Kikuchi et al., 2004). In addition, *Bordetellae bronchiseptica* can alter DC function by affecting DC signalling pathways that control DC maturation, leading to increased bacterial survival within the host (Skinner et al., 2004). Furthermore, the expression of serotype B polysaccharide by *Neisseria meningitides* inhibited adherence to DCs (Kolb-Maurer et al., 2001). Thus, impairment of DC maturation by encapsulated variants may help the bacterium to escape from host immune defence mechanism (Colino and Snapper, 2003). Several bacterial toxins have recently been reported to alter DC function and therefore impair the protective immune response against the bacteria. Anthrax lethal toxin (Agrawal et al., 2003) as well as *Escherichia coli* heat-labile enterotoxin (Petrovska et al., 2003) have been shown to impair DC function and induce an adaptive immune response.

1.2.3.3. Interference with T cell activation

Microbial pathogens can modulate cytokine release by DCs (Tortorella et al., 2000). The production of IL-12 by dendritic cells in the early phase of an adaptive immune response is essential for the development of Th1 cells that mediate host resistance to pathogens (Moll, 2003). Autocrine IL-10 has been shown to down regulate the IL-12 response of dendritic cells after mycobacterial infection, as mannosylated lipoarabinomannans from *M. bovis* and *M. tuberculosis* are able to inhibit IL-12 production by DCs (Demangel et al., 2002; Nigou et al., 2001). Dendritic cells exposed to *Bordetella pertussis* filamentous hemagglutinin are also impaired in their ability to produce IL-12, but release IL-10 and induce antigen-specific regulatory T cells that suppress protective Th1 responses (McGuirk et al., 2002).

1.3. *Staphylococcus aureus*

1.3.1. Bacteriology

The genus *Staphylococcus* belongs to the family *Micrococcaceae*. The organisms are Gram-positive cocci, about 1 μm in diameter, and occur in an irregular grape-like cluster. They are catalase-positive and oxidase-negative. *S. aureus* is a non-motile, non-spore-forming, facultative anaerobe that can grow in medium containing 15% sodium chloride and at the temperature range of 15°C to 45°C. *S. aureus* is distinguished from the remaining staphylococcal species on the basis of the golden pigment of colonies on rich medium and it is positive for coagulase, deoxyribonuclease and mannitol-fermentation (Gillespie, 1994; Murray et al., 1994).

The staphylococcal cell wall (Fig 1-1) has a thick peptidoglycan layer surrounding the cytoplasmic membrane of the cell. Peptidoglycan consists of alternating polysaccharide subunits of *N*-acetylglucosamine and *N*-acetylmuramic acid. Another important component of the cell wall is lipoteichonic acid (LTA). LTA is a glycerol phosphate polymer linked to a glycolipid terminus anchored in the cytoplasmic membrane (Lowy, 1998). These components have endotoxin-like activity, stimulating the release of cytokines by macrophages and DC (IL-1 β , IL-6, IL-8, and TNF), activating the alternative complement pathway, and aggregation of platelets. (Lowy, 1998; Nair et al., 2000) Peptidoglycan and LTA induce DC maturation via TLR2 (Michelsen et al., 2001).

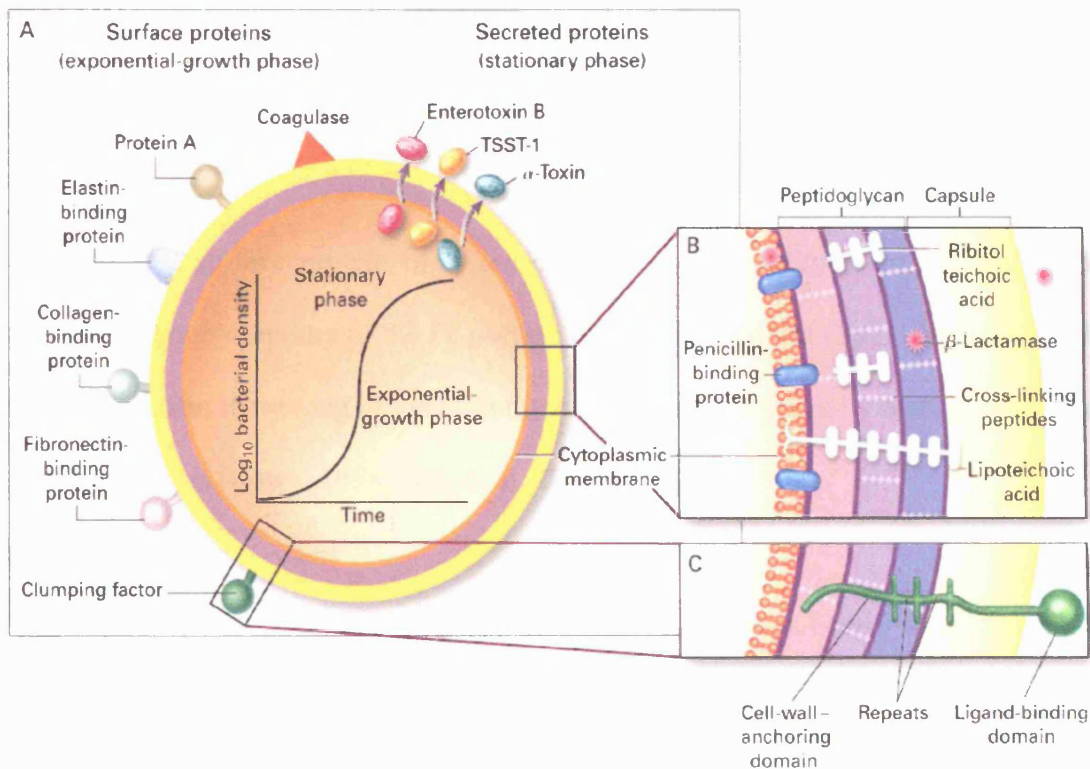


Figure 1-1. *Staphylococcus aureus* structure.

(A) The synthesis of many of surface and secreted proteins is dependent on the growth phase. (B and C) Cross sections of the cell envelope, many of the surface proteins have a structural organization similar to that of clumping factor. Adapted from (Lowy, 1998)

Most strains of *S. aureus* (90%) produce capsular polysaccharide, which forms an outer surface layer on the peptidoglycan in the cell wall. There are 11 different capsular serotypes that can be produced by *S. aureus*, serotypes 5 and 8 are the most common, occurring in 80% of strain isolated from human infections (Lowy, 1998). It has been reported that capsular polysaccharide can enhance staphylococcal virulence by inhibiting phagocytosis (Lowy, 1998; Nair et al., 2000).

Protein A is a surface protein of *S. aureus* that has the ability to help evade the host immune system by binding to the Fc portion of immunoglobulin (IgG). Bacteria will bind IgG molecules in the wrong orientation on their surface and there by disrupt opsonization and phagocytosis (Lowy, 1998; Nair et al., 2000).

Coagulase is an extracellular protein that exists in free and bound forms. It binds to prothrombin in a 1:1 ratio forming a complex called staphylothrombin. The binding reaction catalyses plasma clotting. This clot may provide protection to the bacterium, which may be coated with fibrin and it inhibits phagocytosis. Coagulase is a traditional marker for identifying *S. aureus* in clinical microbiology (McDevitt et al., 1992; Nair et al., 2000).

S. aureus also produces a group of surface proteins that promote attachment to host extracellular matrix as an initial step in the colonization process. These proteins are collectively known as microbial surface components recognizing adhesive matrix molecules (MSCRAMM) (Foster and Hook, 1998). Most of MSCRAMMs are anchored in the cell wall peptidoglycan and have the ability to bind to a variety of host matrix

proteins such as fibronectin, collagen, fibrinogen and elastin (Lowy, 1998; Nair et al., 2000).

The genome of *S. aureus* consists of a circular chromosome (of about 2800bp), with prophages, plasmids, and transposons. Genes involved in virulence and resistance to antibiotics are found on the chromosome and on extra-chromosomal elements (Lowy, 1998). *S. aureus* virulence factor production is growth phase-dependent in liquid culture, most exotoxins are produced in the post-exponential phase of growth and are not required for growth and multiplication of the bacterium. The regulation of virulence factor production in *S. aureus* is controlled by at least two global regulators, *agr* (Accessory Gene Regulator) and *sar* (Staphylococcal Accessory Regulator) (Yarwood et al., 2001).

1.3.2. *Staphylococcus aureus* infections

The organisms normally grow on the skin and/or mucous membranes in 20-50% of the population. These barriers prevent the organisms from gaining access to other body organs and tissues (Nair et al., 2000). Human staphylococcal infections are frequent, but usually remain localized at the portal of entry by the normal host defenses. The portals of entry are usually a break in the skin by needle-stick or surgical wound and the respiratory tract. The localized host response to infection is inflammation, swelling, the accumulation of pus, and necrosis of the tissue (Gillespie, 1994). *S. aureus* is responsible for a number of human diseases such as those affecting the skin: impetigo, pustules, boils, carbuncles, and cellulitis. It is also a common cause of postoperative sepsis, wound infection, abscesses, pyogenic infection (e.g. endocarditis and osteomyelitis), septicaemia, food poisoning and toxic shock syndrome (Dinges et al., 2000; Bergdoll, 1989).

S. aureus causes disease by producing a large numbers of exoproteins or virulence factors (Murray et al., 1994; Bergdoll, 1989). There are two types of exoproteins: (a) enzymatic proteins that may help to provide nutrients for bacteria, such as nucleases, hyaluronidase, proteases, collagenases and lipases; (b) exotoxins that are the causative agents of disease, including toxic shock syndrome toxin-1 (TSST-1), staphylococcal enterotoxins (SEs), exfoliative toxin (ETs), and Panton-Valentine leukocidins (Proft and Fraser, 2003; Bergdoll, 1989). Some exoproteins, for instance the hemolysins (α , β , γ , and δ) possess both enzymatic and toxin activities (Murray et al., 1994).

1.3.3. Pyrogenic exotoxin as superantigens

1.3.3.1. Superantigens: definition and mechanism of immune stimulation

White et al (1989) (White et al., 1989) described a group of microbial antigens that are able to activate a large fraction (5-20%) of the resting T-lymphocyte population, compared to the small numbers (one in 10^5 - 10^6 T-cells) activated with conventional proteins or peptide antigens. These were termed superantigens (SAGs) (Table 3). T-lymphocytes can recognize a wide variety of antigens via highly diverse cell-surface glycoproteins known as T cell receptors (TCR). These disulfide-linked heterodimers are composed of α and β or γ and δ chains that have variable (V) and constant (C) regions that are very similar in structure of the Fab (antigen binding) fragment of an immunoglobulin molecule. Unlike antibodies, which recognize antigen alone, TCRs recognize antigen only in the form of peptide bound to major histocompatibility complex (MHC) molecules (Henderson et al., 1999; Proft and Fraser, 2003).

Table 1-3: Comparison of antigen, superantigen, and mitogen

Feature		Antigen	Superantigens	Mitogen
Percentage of stimulated cells		0.0001-0.000001	5-20	80-90
Accessory cell requirement		✓	✓	✓
Dependence on MHC-II expression		✓	✓	✗
Antigen processing		✓	✗	✗
Restricted T cell V β usage by stimulating cells		✓/(✗)	✓	✗

(Kotb, 1995; Henderson et al., 1999)

Normally, antigen presenting cells (APC) break down antigens into small peptides, which form complexes with MHC class II molecules. These complexes are transported to the cell surface (MHC peptide-binding groove) to bind with $\alpha\beta$ -TCR (Kotb, 1995). SAgS bind to MHC class-II on APCs (B-cells, monocytes, and dendritic cells) and are recognized by TCRs, but in a different way than MHC class-II-antigen complexes are recognized. The main characteristic of superantigens is that they do not need processing by the APCs and they can activate T-cells by binding with the variable region of the β chain of TCR ($V\beta$) (Henderson et al., 1999; Proft and Fraser, 2003; Marrack and Kappler, 1990).

The cross-linking between T-cells and SAgS does not always lead to activation and proliferation of T-cells, but responding cells can either enter a state of unresponsiveness termed T-cell anergy, or in the presence of high level of tumor necrosis factor- α (TNF- α), and interferon- γ (IFN- γ) they become apoptotic (Henderson et al., 1999; Kotb, 1995; Proft and Fraser, 2003). SAg binding induces both the APC and T lymphocyte to produce a variety of cytokines including, interleukins-1 (IL-1), TNF- α , IL-2, TNF- β and IFN- γ (Fig 1-2). This dramatic activation causes toxic shock syndrome (Le Loir et al., 2003).

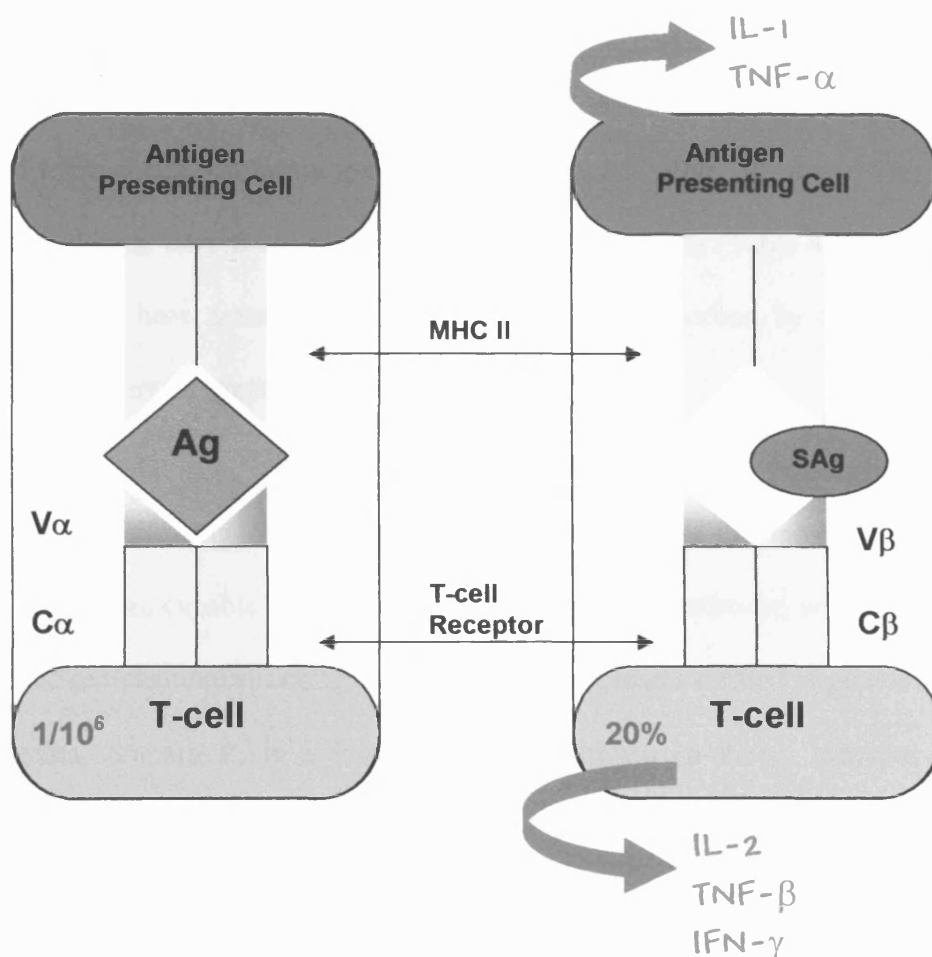


Figure 1-2: Schematic diagram showing the superantigen (SAg) interaction with MHC class II and TCR.

The processed antigen (Ag) peptide presented by MHC class II which binds specific T cell bearing antigen specific TCR. In contrast, SAg binds directly to the MHC molecule outside the antigen binding site and cross-links it to the Vβ chain, which initiates non-specific activation of T cells and induces massive production of cytokines.

1.3.3.2. *Staphylococcus aureus* superantigens

The known staphylococcal pyrogenic superantigens are the staphylococcal enterotoxins (SEs) and toxic shock syndrome toxin-1 (TSST-1) (Kotb, 1995; Proft and Fraser, 2003). SAGs secreted by *S. aureus* are globular proteins of 22-29 KDa (Table 4) that are resistant to proteases and heat denaturation and are able to be absorbed by the epithelium as immunologically intact proteins (Hamad et al., 1997).

SAGs have the following characteristics, (a) they are among the most potent pyrogens known, (b) they are capable of inducing lethal toxic shock syndrome, and (c) they share a typical three-dimensional structure consisting of two domains termed large and small. The small domain, domain B, is a β -barrel made up of two β -sheets, whereas the large domain, domain A, contains a β -grasp motif and an α -helix packed against a mixed β -sheet that connects the peripheral strands (Li et al., 1999; Prasad et al., 1993; Proft and Fraser, 2003; Swaminathan et al., 1992).

The level of sequence homology between the staphylococcal SAGs varies widely, and they can be divided into groups based on sequence similarities. SEA, SED and SEE would fall into one group (53-81%) and SEB and SECs (50-66%) would fall into another. All the rest, including TSST-1, have poor or no homology to another toxin (Kotb, 1995; Li et al., 1999; Proft and Fraser, 2003).

The interaction of SAgS with MHC class II molecules appears to be involved more than one binding site and varies among these toxins (Proft and Fraser, 2003). Both SEB and TSST-1 have a hydrophobic loop region in the smaller N-terminal domain which binds to a hydrophobic groove of HLA-DR (Prasad et al., 1993; Swaminathan et al., 1992). However, the structures of these toxins are not identical. SEB binds out to the side of the MHC II peptide binding groove while TSST-1 sits over the top of the groove and makes contacts with the peptide residues. According to this feature, TSST-1 prevents any contact between MHC II and the TCR while SEB relies on continued contacts between MHC and TCR to strengthen the interaction (Li et al., 1999; Papageorgiou et al., 1995; Proft and Fraser, 2003). The affinity of SAgS towards MHC class II also varies. SEB and TSST-1, bind with relatively low affinity, while other SAgS, such as SEA and SEE, have high-affinity zinc-mediated binding sites for the polymorphic HLA-DR (Dinges et al., 2000; Kotb, 1995; Le Loir et al., 2003; Proft and Fraser, 2003).

Table 1-4: *Staphylococcus aureus* superantigens

Toxin	Molecular mass (KDa)
SEA	27.1
SEB	28.4
SEC1	27.5
SEC2	27.6
SEC3	27.6
SED	26.9
SEE	26.8
SEG	27.0
SEH	25.2
SEI	24.9
SEJ	28.5
SEK	25.3
SEL	24.7
SEM	24.8
SEN	26.1
SEO	26.7
SEP	26.4
SEQ	26.0
TSST-1	21.9

(Lina et al., 2004; Proft and Fraser, 2003)

1.3.3.2.1. Staphylococcal Enterotoxins (SEs):

These proteins are heat stable exotoxins that are known for causing one of the common food-borne illnesses, food poisoning (intoxication), associated with ingestion of toxin-contaminated food (Orwin et al., 2001). This contamination is usually linked to poor hygiene and improper storage of food (Bean et al., 1996). Most cases are self-limiting after 24 hours of ingestion. In the first 4 hours patients may have diarrhea, nausea, vomiting and abdominal pain or cramping. Eighteen staphylococcal enterotoxins have been described in the literature, which share structure and sequence similarities (Table 4) (Lina et al., 2004; Proft and Fraser, 2003; Orwin et al., 2003).

Enterotoxin activity is uniquely characterized by the ability to cause an emetic response when administered orally to humans and primates, whilst other superantigens are not emetic (Dinges et al., 2000). The infective dose required to induce staphylococcal food poisoning in human is approximately 0.1 µg, and it depends on patient sensitivity (Evenson et al., 1988). Although there are many reports on the structure-function relationship of superantigen activity of SEs, the emetic activity has not been fully documented. One common structural characteristic of SEs is a cystine loop, suggested to be involved in emetic activity (Dinges et al., 2000). However, SEI lacks that loop structure and has both emetic and superantigenic activities (Munson et al., 1998). SEK and SEL are recently identified superantigens in which the cystine loop is missing (Fitzgerald et al., 2001; Orwin et al., 2003). These later SEs have not been tested for their emetic activity yet (Le Loir et al., 2003).

1.3.3.2.2. Toxic Shock Syndrome Toxin-1 (TSST-1):

Toxic shock syndrome (TSS) is a disease caused by *Staphylococcus aureus* and characterized by fever, rash, hypotension and multiple organ failure. TSS cases are associated with strains of *S. aureus* that produce TSST-1 (Murray et al., 1994; Proft and Fraser, 2003). TSST-1 is a potent superantigen that interacts with human T-cells via V β 2 elements (Dinges et al., 2000), and induces the production of excessive amounts of inflammatory cytokines (Dinges et al., 2000; Kotb, 1995).

1.3.4. Staphylococcal Superantigen-Like proteins (SSL):

Staphylococcal superantigen-like (SSL) proteins (previously staphylococcal exotoxin-like proteins (SET)) are a group of proteins produced by *S. aureus* that contain SAg signature sequences. They were first identified as a genetic locus encoding at least five exotoxin-like proteins (SET1-5), and were reported to have the capability to stimulate the secretion of some proinflammatory cytokines, such as IL-1 β , IL-6 and TNF- α , by human peripheral blood mononuclear cells (PBMCs) (Williams et al., 2000). More recently, the whole genome sequence of different *S. aureus* strains has revealed a large number of related (36-67%) *set* genes on the SaPI₂ pathogenicity island of *S. aureus* genome (Baba et al., 2002; Kuroda et al., 2001). There appears to be extensive inter-strain allelic polymorphism for each of the *set* genes (Baba et al., 2002; Fitzgerald et al., 2003; Kuroda et al., 2001) (Fig 1-3 and 1-4).

It has recently been reported, that members of this family of proteins do not have the main properties of superantigens, such as superantigenicity, pyrogenicity, and enhancement of endotoxin shock (Arcus et al., 2002; Fitzgerald et al., 2003). Arcus and colleagues (Arcus et al., 2002) determined the first three-dimensional structure of a protein, SET3, belonging to this family. The crystal structure of this protein shows the characteristic structure of the superantigen super-family, however, this protein family may have a non-redundant biological function that is completely different from that of the superantigens.

The International Nomenclature Committee for Staphylococcal Superantigen Nomenclature (INCSS) has recently recommended that the SETs be renamed staphylococcal superantigen-like proteins (SSLs) and should be named *ss/1* to *ss/11* in clockwise order from the replication origin of the chromosome based on homology to the full complement of genes found in strain MW2 (Lina et al., 2004). This nomenclature is essentially as described by Fitzgerald et al (Fitzgerald et al., 2003) except that the numbering of the genes is in the opposite direction (Fig 1-3). To differentiate between allelic variants the *ss/* gene is prefixed by the strain name. This nomenclature is used throughout this thesis.

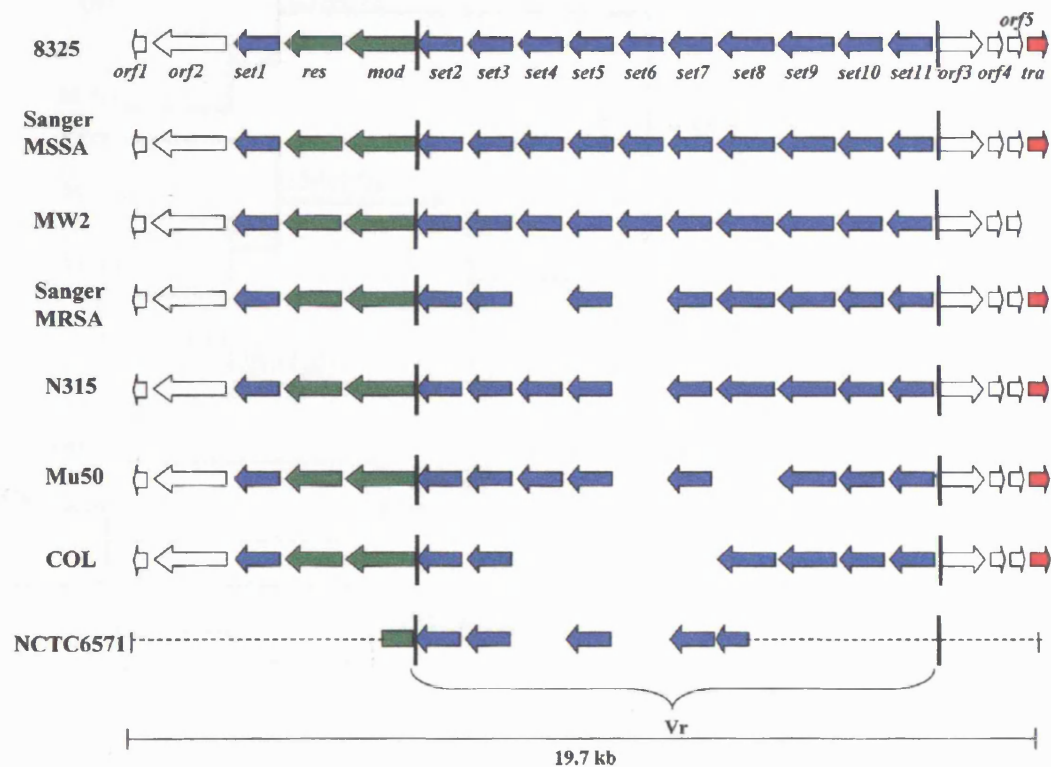


Figure 1-3. The SET proteins encoded by the genes designated as *set1* to *set11*; in eight *S. aureus* strains.

Comparison of chromosomal regions from different *S. aureus* strains; 8325, Sanger MSSA, MW2, Sanger MRSA, N315, Mu50, COL, and NCTC6571, indicates that the *set* genes in different strains are allelic variants of each other. The SETs were renamed staphylococcal superantigen-like exoproteins (SSLs) using the numbering system SET(x) introduced by Fitzgerald et al (Fitzgerald et al., 2003); but *ss1* to *ss11* in clockwise order from the replication origin of the chromosome based on homology to the full complement of genes found in strain MW2 (Lina et al., 2004). Adapted from (Fitzgerald et al., 2003).

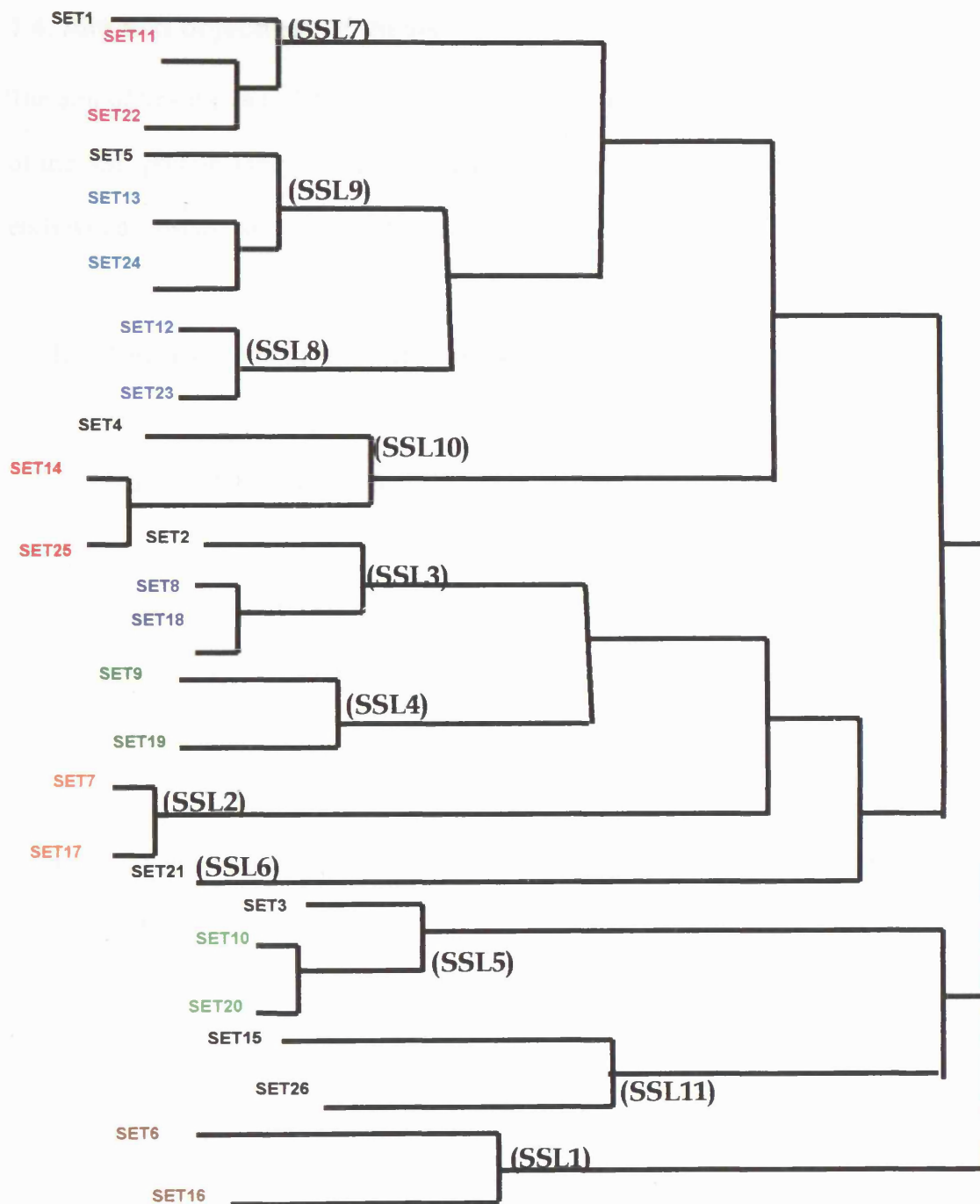


Figure 1-4: Phylogenetic tree showing the relationship between the members of SSL (SET) family.

The SETs renamed staphylococcal superantigen-like exoproteins (SSLs) using the numbering system SET(x) introduced by Fitzgerald et al (Fitzgerald et al., 2003) but in the opposite direction.

1.4. Aim and objectives of thesis

The aim of this thesis is determination and understanding of the immunological functions of the SSL protein family. The investigation will be divided into three main objectives; each will be discussed in a separate chapter:

1. Characterisation of the interaction between SSL proteins and cells of the immune system; the cell populations which bind to these proteins and the features of this binding will be investigated.
2. The influence of SSL proteins on DC functions such as endocytosis and antigen presentation, as well as the immunological responses toward these secreted proteins in the normal human population will be assessed.
3. Biochemical methods will be used to identify the SSL receptor(s) from selected cell lines.

CHAPTER II

Material and Methods

2. Materials & Methods

2.1 Molecular biology

2.1.1 Cloning and purification of recombinant proteins

2.1.1.1 *Cloning of ssIs and embp32 genes into the pQE30 vector*

The *ssIs* genes (*ssl4*, *ssl5*, *ssl7*, *ssl9* and *ssl10*) from *S. aureus* NCTC 6571 and *embp32* gene from *S. epidermidis* NCTC 11047 were cloned into pQE30 (Qiagen Ltd) an N-terminal histidine tag fusion vector and introduced into *E. coli* M607 (pREP4), by Dr. Rachel Williams, Division of Microbial Diseases, Eastman Dental Institute, UCL. Investigations into the conditions for optimal expression of rSSL proteins revealed that SSL7 and SSL9 were expressed in highest amounts, and these two proteins were therefore chosen for further study.

2.1.1.2 *Expression and purification of recombinant proteins*

Clones were grown overnight at 30°C with shaking in 20 ml Luria-Bertani (LB) broth containing 100 µg/ml ampicillin and 25 µg/ml kanamycin. The overnight cultures were used to inoculate 200 ml of fresh medium and incubated at 37°C for 2 hours. Gene expression was induced with 200 µl of 1M isopropyl-B-D-thiogalactopyranoside (IPTG) for 4 hours at 30°C. Cells were harvested by centrifugation at 6,000 X g at 4°C for 20 minutes in a Sorvall RC5B centrifuge, and stored at -20°C overnight.

Cells were harvested and lysed for 30 minutes at 4°C in 4 ml B-PER (Bacterial Protein Extraction Reagent, Pierce & Warriner LTD.) containing 20 mM imidazol, 40 µl of 120 mM PMSF (phenylmethyl-sulfonyl fluoride) and 4 µl each of 1 mM E64, 10 mM leupeptin and 1 mM peptatin as protease inhibitors. The lysate was clarified by centrifugation at 23,000 X g for 10 minutes. Recombinant proteins were purified using Ni-nitrilotriacetic acid-agarose columns (Ni-NTA, Qagen Ltd.). The columns were washed with B-PER and PBS containing 20 mM imidazol. 2.5 mg of polymixin B per ml in wash buffer was loaded onto the column to remove contamination with lipopolysaccharide (LPS). Recombinant proteins were eluted from the column with PBS containing 250 mM imidazole. Finally, rSSLs and rEmbp32 were subjected to dialysis using Slide-A-lyzer cassettes (Pierce) against PBS buffer to remove imidazol. All rSSL proteins were homogenous as determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and migrated with molecular masses of between 23 to 27 kDa (Fig. 2-1)

2.1.2 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed using a Mini PROTEAN II electrophoresis tank (Bio-RAD). The separating gel (8cm x 7cm) was made with 0.4M Tris-HCl pH 8.8, 0.1% (w/v) SDS and 12% (w/v) acrylamide mix [29.2% (w/v) acrylamide, 0.8% (w/v) bisacrylamide; National Diagnostic, UK], 50µl of 10% ammonium persulphate (APS) and 5µl of TEMED (N,N,N',N'-tetramethylethylenediamine) per 5ml gel were added, to give 12% gel. 10% gels were performed in same way but by adding 10% acrylamide mix (w/v). Once poured into the gel apparatus, it was overlaid with distilled water to produce a flat surface to the gel and allowed to set for 45 minutes at room temperature.

The stacking gel was then prepared and poured on top of the separating gel. This 5% gel was made with 0.125M Tris-HCl pH 6.8, 0.1% (w/v) SDS, 3.9% (w/v) acrylamide mix, and 50µl 10% APS and 5µl TEMED were added per 5ml gel. A comb was inserted after pouring the gel to allow wells to form for sample application. The gel was left to set for 30 minutes at RT before the comb was removed and the gel mounted onto the electrophoresis apparatus. Samples were mixed with 5X reducing sample buffer (Pierce, UK) and boiled at 95°C for 5 minutes prior to loading on the gel. SDS-PAGE running buffer (25mM Tris-HCl pH 8.3, 250mM glycine, 0.1% SDS) was added to the apparatus. Samples and broad range molecular mass markers (New England Biolabs, UK) were electrophoresed through the stacking gel at constant current of 30 mA per gel. Once the samples had passed through the stacking gel the current was reduced to 15 mA per gel until the dye front reached the bottom of the gel.

2.1.2.1 Staining SDS-PAGE gels

Gels were first fixed for one hour with 40% (w/v) methanol, 15% (v/v) acetic acid at room temperature (RT). Colloidal Brilliant blue concentrate (Sigma) was made up according to manufacturer instruction and four parts of stain were mixed with one part of methanol immediately prior to use. The gels were soaked overnight in the stain before destaining in water

2.1.3 Cloning of the *ssl7* gene into the pGEX vector

In order to produce recombinant SSL7 protein without the histidine tag, a cleavable affinity tag (GST) expression vector pGEX-41-1 on a *Bam*III-*Sal*I fragment and subcloned into *E. coli* JM109.

2.1.3.1 Plasmid DNA isolation

The plasmid pQE30 containing NCTC6571 *ssl7* gene was purified from 10 ml of overnight culture using the QIAquick plasmid mini-prep kit (Qiagen) according to the manufacturer's instructions. Plasmid DNA was stored at -20°C.

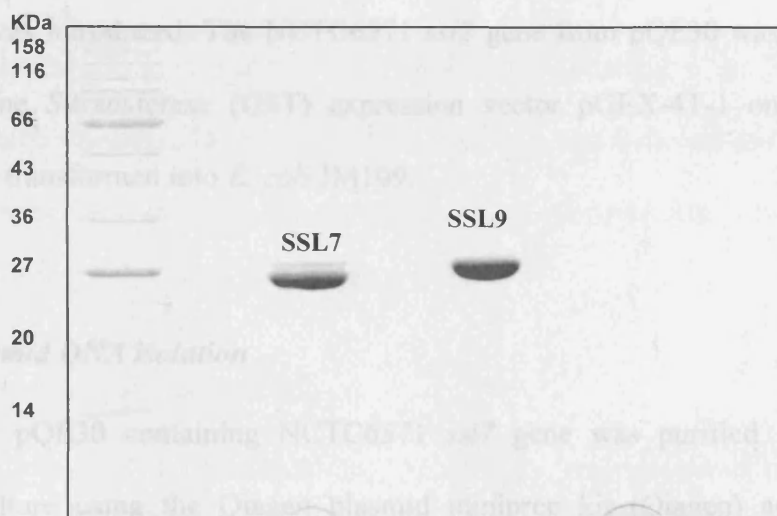


Figure 2-1. Purification of SSL7 and SSL9 proteins by Ni-NTA affinity chromatography.

The *ssls* genes (*ssl7* and *ssl9*) from *S. aureus* NCTC 6571 were cloned into pQE30 an N-terminal histidine tag fusion vector and introduced into *E. coli* M607 (pREP4). SSL7 and SSL9 proteins were expressed and purified using Ni-NTA columns. The columns were washed with B-PER and PBS containing 20 mM imidazol. Polymixin B was loaded onto the column to remove contamination with lipopolysaccharide (LPS). Recombinant proteins were eluted from the column with PBS containing 250 mM imidazole. The figure is a photograph of Colloidal Brilliant blue stained 12% SDS-PAGE. The lane and numbers on the left represent molecular mass standards in kilodaltons.

2.1.3 Cloning of the *ssl7* gene into the pGEX vector

In order to produce recombinant SSL7 protein without the histidine tag, a cleavable affinity tag was introduced. The NCTC6571 *ssl7* gene from pQE30 was subcloned into the glutathione *S*-transferase (GST) expression vector pGEX-4T-1 on a *Bam*HI-*Sal*I fragment and transformed into *E. coli* JM109.

2.1.3.1 Plasmid DNA isolation

The plasmid pQE30 containing NCTC6571 *ssl7* gene was purified from 10 ml of overnight culture using the Qiagen plasmid miniprep kit (Qiagen) according to the manufacturer's instructions. Plasmid DNA was stored at -20°C.

2.1.3.2 Agarose gel electrophoresis

To make 1% (w/v) agarose gel, agarose powder was weighed out and dissolved in Tris-Borate EDTA (TBE) buffer (90mM Tris-HCl, 90mM boric acid, 1mM EDTA) by heating in a microwave. After the solution had cooled to less than 50°C, ethidium bromide was added to a final concentration of 0.5µg/ml and the mixture poured into a horizontal casting tray. The tray also held a template comb to form loading wells. The gel was then left for 30 minutes to set at room temperature. The gel was placed in an electrophoresis tank (Biometra) containing TBE and the comb was removed gently. The samples to be analysed were mixed (5:1) with 6x gel loading buffer (0.25% bromophenol blue, 40% sucrose, 0.1M EDTA). Samples and markers (New England Biolabs) were loaded into the gel wells and electrophoresis was performed at 50V for 30-60 minutes. The plasmid DNA was visualised under UV light (365nm) and photographed using a multiImageTM Light

Cabinet with AlphaImager™ 1220 documentation and analysis system (Alpha Innotech Corporation, UK)

2.1.3.3 Isolation of DNA from agarose gels

DNA bands of interest were excised from the agarose gel using a sterile scalped blade, and the gel slices transferred to a sterile tube. The DNA was then isolated from the gel by using the Qiagen Gel Extraction Kit, according to the manufacturer's instructions. DNA was stored at -20°C.

2.1.3.4 Restriction enzyme digestion

Digestion of plasmid pQE30 containing NCTC6571 *ssl7* gene and pGEX-4T-1 was carried out using *Bam*HI and *Sal*I restriction endonucleases. Plasmid was incubated with 1 µl of restriction enzymes (6-12U) and 2 µl of the appropriate 10x buffer in a total volume of 20µl at 37°C for 4 hours.

2.1.3.5 Ligation of the *ssl7* gene to the pGEX-4T-1 vector

1 µg of restriction enzyme-digested plasmid and *ssl7* gene were added to a tube with 2 µl of 10x ligation buffer and 5U of ligase enzyme (New England Biolabs). The mixture was made up to a final volume of 15µl and incubated overnight at 4°C.

2.1.3.6 Preparation of competent cells

A single colony of *E. coli* JM109 was used to inoculate 5ml of LB medium and incubated overnight at 37°C with shaking. The next day 60 µl of the overnight culture was used to inoculate 10 ml of fresh LB medium. The bacteria were grown at 37°C with shaking to reach an absorbance at 600nm of 0.5. The cells were then harvested by centrifugation at 4,000 X g for 15 minutes at 4°C in an Eppendorf 5804R centrifuge. Cells were

resuspended in 5ml of ice cold 75mM CaCl₂, 15% glycerol. The cells were harvested by centrifugation at 4,000 x g for 15 minutes at 4°C. The supernatant was discarded and the cells were resuspended in 1 ml of ice cold 75mM CaCl₂, 15% glycerol. The cells were divided into 150µl aliquots and stored at -70°C immediately.

2.1.3.7 Transformation of competent *E. coli*

Ligation mix (10 µl) was added to competent *E. coli* JM109 cells suspension (150 µl), mixed and incubated for 45 minutes on ice. The cells were heat-shocked at 42°C for 30 seconds, and returned to ice for an extra 2 minutes. LB broth (1 ml) was added to the cells and incubated for a further 1 hour at 37°C with shaking. Cell suspensions were centrifuged at 5000 rpm for 10 minutes, then 100 µl of LB medium were mixed with the pellet and plated overnight on LB agar containing 100 µg/ml ampicillin.

2.1.3.8 Western blotting

In order to check the conditions for optimal expression of rSSL7 protein, small scale cultures were prepared. Positive clones were cultured (10 ml) and induced with a range of concentrations of IPTG (0, 0.25 and 1M) at 30°C or 37°C and samples taken every 2 hours after incubation. Cells were harvested and resuspended in 100 µl B-PER containing proteases inhibitors (Sigma). The supernatants were clarified by centrifugation at 5000 rpm for 10 minutes and separated by 12% SDS-PAGE.

The gel was removed from the electrophoresis tank and soaked in blotting buffer (28.8g glycine, 6g Tris-HCl, 400ml methanol, made up to 2 liters with dH₂O) for 1 hour. Immobilon-P polyvinylidene fluoride (PVDF) membrane was then placed on top of the gel and sandwiched between eight pieces of blotting papers. The sandwich was placed into a Mini PROTEAN II electrophoresis tank submerged in blotting buffer. The tank was then subjected to a constant voltage of 15V for 16 hours. Membranes were removed from

the tank and soaked in wash buffer (PBS containing 0.1% (v/v) Triton X-100) for 10 minutes, then incubated in blocking buffer (PBS, 0.1% (v/v) Triton X-100, 5% (w/v) skimmed milk) for 1 hour. Afterward, the membranes were incubated with goat anti-GST antibodies (Amersham), diluted appropriately in blocking buffer, for 2 hours. The membranes were washed 5 times before being incubated with detection antibody, a horseradish peroxidase (HRP) conjugate (Sigma) diluted in blocking buffer, for 1 hour. The membranes were washed 5 times with wash buffer before developing with diaminobenzidine (DAB, Sigma) until the bands could be seen. The membranes were dried, photographed and stored in a dark place to prevent the bands from fading.

Once the best conditions for expression were established, larger cultures were induced to allow protein isolation.

2.1.3.9 Expression and purification of rSSL7 protein

For gene expression, an overnight culture was diluted 1:25 in fresh LB broth containing 100 µg/ml ampicillin and incubated for 2 h at 37°C. Gene expression was induced with 1 mM IPTG for 4 h at 37°C. Cells were harvested by centrifugation at 6,000 X g for 30 min, resuspended, and lysed for 20 min in B-PER protein extraction reagent (Pierce & Warriner Ltd.) containing protease inhibitors (Sigma). Lysates were clarified by centrifugation at 23,000 X g for 10 min. The lysate containing the GST-SSL7 fusion protein was passed through a HiTrap GST-Sepharose column (Amersham-Pharmacia Biotech), and recombinant SSL7 without the GST tag was released from the column by digestion with thrombin essentially as described by the supplier of the column (Amersham-Pharmacia Biotech). SSL7 protein was homogenous as determined by SDS-PAGE and migrated with molecular masses of about 24 kDa (Fig. 2-2)

2.1.4 FITC labelling of SSLs

SSL7 and SSL9 were dialysed against labelling buffer (0.2 M NaHCO₃, pH 9.0) overnight at room temperature (RT). 50 µl of 1 mg/ml fluorescein isothiocyanate (FITC, Sigma) in dimethyl sulfoxide (DMSO) was added to 1 ml of a 2 mg/ml protein solution. After 4 h incubation at RT in the dark, unbound FITC was removed by size exclusion

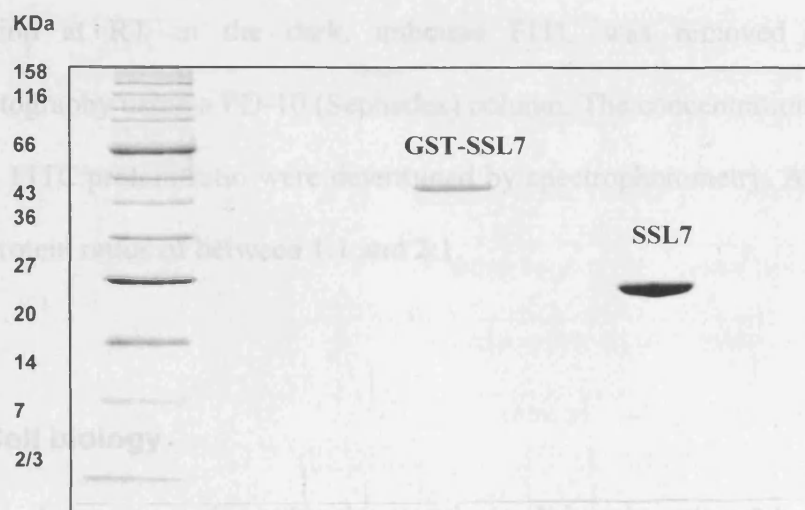


Figure 2-2. Purification of SSL7 protein by GST affinity chromatography.

The NCTC6571 *ssl7* gene from pQE30 was subcloned into the glutathione *S*-transferase (GST) expression vector pGEX-4T-1 and was transformed into *E. coli* JM109. Gene expression was induced with 1 mM IPTG, cells then were harvested by centrifugation. The lysate containing the GST-SSL7 fusion protein (50 KDa) was passed through a GST-Sepharose column, and recombinant SSL7 without the GST tag was released from the column by digestion with thrombin. The figure is a photograph of Colloidal Brilliant blue stained 12% SDS-PAGE. The lane and numbers on the left represent molecular mass standards in kilodaltons.

2.1.4 FITC labelling of SSLs

SSL7 and SSL9 were dialysed against labelling buffer (0.2 M NaHCO₃, pH 9.0) overnight at room temperature (RT). 50 µl of 1 mg/ml fluorescein isothiocyanate (FITC, Sigma) in dimethyl sulfoxide (DMSO) was added to 1 ml of a 2 mg/ml protein solution. After 4 h incubation at RT in the dark, unbound FITC was removed by size exclusion chromatography using a PD-10 (Sephadex) column. The concentration of labelled protein, and the FITC:protein ratio were determined by spectrophotometry. All preparations gave FITC:protein ratios of between 1:1 and 2:1.

2.2 Cell biology

All cell culture was performed under aseptic conditions in a class 2 laminar air flow safety cabinet, using sterile techniques. Cells were maintained by incubation at 37°C in an atmosphere of 5% CO₂/95% Air.

2.2.1. Cell lines

Cells were maintained in complete medium (CM) (RPMI 1640 medium (Gibco) supplemented with 10% fetal calf serum (FCS; PAA Laboratories), 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine (Clare Hall Laboratories, Imperial Cancer Research Fund), and split twice a week.

Table 2-1 Cell lines used in this study

Cell lines	Description
K562	Human erythroleukemic cell line
HL-60	Human promyelocytic leukemia cells
THP-1	Human acute monocytic leukemia cells
U937	Human monocytic leukaemia cell line
KG1	Human acute mylogenous leukaemia
WMPT	EBV-transformed human B cell lines
WMPT 3.3	EBV-transformed human B cell lines
Fc7	EBV-transformed human B cell lines

2.2.2. Cell viability

Cell viability was assessed by Trypan Blue exclusion assay. 10 µl of cell suspension was diluted in an equal volume of 0.04% Trypan Blue solution. 10 µl of this mix was loaded on a haemocytometer counting chamber (Neubauer) placed under the microscope and white live cells (dead cells turn blue) were counted within a 4 x 4 square grid.

2.3 Cellular immunology

2.3.1 Antibodies

The following monoclonal antibodies (MAbs) were used: CD2 (mouse MAb MAS 593, IgG_{2b}; Harlan), CD3 (supernatant mouse MAb UCHT1, IgG₁; gift from P. C. L. Beverley [Edward Jenner Institute for Vaccine Research, Compton, UK]), CD14 (supernatant mouse MAb HB246, IgG_{2b}; gift from P. C. L. Beverley), and CD19 (supernatant mouse MAb BU12, IgG₁; gift from D. Hardie [Birmingham University, Birmingham, UK]), HLA-DR (supernatant mouse MAb L243, IgG_{2a}; gift from P. C. L. Beverley), HLA-ABC (W6/32; Serotec), CD86 (supernatant mouse MAb BU63, IgG₁; gift from D. Hardie), and CD54 (Mouse IgG MEM-111, gift from Prof. Horejsi, Academy of Science, Prague, Czech Republic). Fluorescein isothiocyanate (FITC) conjugated anti-mouse rabbit polyclonal antibody was purchased from Dako. PE-conjugated anti-CD1a (Monoclonal Mouse IgG1, clone BL6, Immunotech, Marseille, France). A rabbit polyclonal antibody to His-SSL7 was produced under contract by Eurogentec Ltd (Southampton, UK) and validated by Western blot and ELISA.

2.3.2 Dendritic cell preparation

All blood samples were collected from healthy volunteers after informed consent had been obtained, following approved departmental safety protocols. The project (93/2684) was approved by the UCLH Ethics committee.

Human peripheral blood mononuclear cells (PBMC)-derived dendritic cells (DC) were generated from fresh whole blood samples obtained from healthy volunteers (Alderman et al., 2002; Newton et al., 2003).

2.3.2.1 Day 1

60 ml of blood containing anticoagulant (heparin), were diluted by an equal volume of Hank's Buffered Saline Solution (HBSS). 30 ml of diluted blood were carefully layered onto 17.5 ml of Lymphoprep (Nycomed Pharma) in a 50 ml tube (Falcon) and centrifuged at 400 X g (1600rpm), at room temperature (RT). After centrifugation for 30 minutes, mononuclear cells in the interface were removed, diluted in HBSS and centrifuged at 400 g for 10 minutes at RT. Cells were pooled, washed twice in HBSS and centrifuged at 1400 rpm for 5 minutes at RT. The cell pellet was resuspended in 10 ml red blood cell (RBC) lysis buffer (Sigma) for 5 minutes at RT, to remove contaminating RBC. Then the cells were washed twice in HBSS, and the pellet was resuspended in 18 ml of complete medium (CM) (RPMI 1640 medium (Gibco) supplemented with 10% fetal calf serum (FCS; PAA Laboratories), 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine (Clare Hall Laboratories, Imperial Cancer Research Fund). Cells were incubated in 6-well tissue culture plates for 2 h at 37°C in 5% CO₂. Non-adherent cells (T and B cells) were removed, washed and centrifuged at 1400 rpm for 5 minutes. The cell pellet was resuspended in 3 ml of 90% FCS: 10% dimethylsulphoxide (DMSO) mix, aliquoted and stored at -70°C until future use.

The adherent cells were cultured in 3 ml fresh CM with 100 ng/ml human recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF) and 50 ng/ml interleukin (IL)-4 (Schering-Plough Research Institute).

2.3.2.2 Day 4

6 ml of loosely adherent cells were collected, and carefully layered onto 4 ml of Lymphoprep in a 15 ml tube (Falcon) and centrifuged at 400 X g (1600rpm), at RT for 30 minutes. Cells from the interface were removed, washed 3 times in HBSS and centrifuged as described above. Contaminating T and B lymphocytes were removed by incubation with CD3, CD2, and CD19 MAbs for 30 minutes on ice. The cells were washed in cold HBSS, resuspended in 2 ml of CM and mixed with anti-mouse IgG-coated immunomagnetic Dynabeads (Dyna) at ratio of 10 μ l beads/10⁶ contaminating cells and incubated on a rotor mixer at 4°C, for 45 minutes. DCs were purified by removing the supernatant after placing the cell suspension/magnetic bead mix in contact with magnet for 10 minutes. The supernatant, containing highly purified DC was cultured for another 3 days at concentration of 1x10⁶ cells/ml in fresh CM containing GM-CSF and IL-4 as described above.

2.3.3 T cells isolation

T cells were frozen at -70°C as described above on day 1 of cell preparation. On day 8, cells were defrosted quickly, washed twice in HBSS and centrifuged at 1400 rpm for 5 minutes at RT. Monocytes and B cells were depleted by incubation with CD19, HLA-DR and CD14 MAbs, for 30 minutes on ice. The cells were washed in cold HBSS, resuspended in 2 ml of CM and mixed with anti-mouse IgG-coated immunomagnetic Dynabeads (Dyna) at ratio of 10 μ l beads/10⁶ contaminating cells and incubated on a rotor mixer at 4°C, for 45 minutes. T cells (greater than 90% purity as measured by expression of CD3, and lack of B cell, NK or monocyte markers) were recovered from the cell suspension after placing of suspension/magnetic bead mix in contact with a magnet for 10

minutes. This method also removed 10% of T cells that express HLA-DR and therefore are considered to be activated (Pollara et al., 2003).

2.3.4 T cell proliferation assay

2.3.4.1 Protocol 1

DCs were serially diluted and incubated at 37°C with autologous T cells (10^5 cells/well) with or without 10 µg/ml SSL proteins in flat-bottomed 96-well microtiter plates. 500 U/ml recall antigen purified protein derivative (PPD) of *Mycobacterium tuberculosis* (Evans Medical) or 10µg/ml of Tetanus toxoid (TT) were used as positive controls. The cell cultures were incubated for 6 days at 37°C, in a 5% CO₂. Cells were pulsed with 1 mCi ³H-thymidine (ICN Biomedical) for 16 h and harvested. T cell proliferation was measured by liquid scintillation counting for ³H-thymidine incorporation. All assays were performed in triplicate and the results are expressed as counts per minutes (cpm).

2.3.4.2 Protocol 2

DCs (10^4) were incubated for 6 days with autologous T cells (2×10^5) in presence of different concentrations of SSL7 or SSL9 (0.42, 1.25 and 4.16 µM) in flat-bottomed 96-well microtiter plates. 500 U/ml recall antigen purified protein derivative (PPD) of *Mycobacterium tuberculosis* (Evans Medical) was used as positive control. The cell cultures were incubated at 37°C, 5% CO₂.

To study the effect of SSL proteins on presentation capacity of DC, purified DCs (10^4), either untreated or treated for 18 hours with different concentrations of SSL proteins (4.16, 1.25 and 0.42 µM), were incubated at 37°C/5% CO₂ with autologous T cells (2×10^5

cells/well) in the presence PPD or with allogeneic T cells in flat-bottomed 96-well microtiter plates. The DC autologous and allogeneic T-cell cocultures were incubated for 6 days. All assays were then pulsed with 1 μCi of [^3H]thymidine (ICN Biomedical, High Wycombe, United Kingdom) for the final 16 h of culture. Cells were harvested, and T cell proliferation was measured by liquid scintillation counting (Microbeta Systems). All assays were performed in triplicate. Results were expressed as cpm. Error bars represent the SD.

2.3.5 Cytokines assays

Autologous T and DCs were incubated with different concentrations of SSL proteins (4.16, 1.25 and 0.42 μM) at 37°C/5% CO_2 in 24-well plates. After 4 days, cell culture supernatants were centrifuged for the removal of cells and stored at -70°C . Cytokine detection was done by enzyme-linked immunosorbent assay (ELISA) for interleukin-10 (IL-10, Pharmingen, UK), gamma interferon ($\text{IFN-}\gamma$, Pharmingen, UK) and IL-13 (ImmunoTools, Germany). Purified protein derivative (PPD; 500 U/ml) was used as a positive control.

2.3.6 Macrophage cell preparation

Human PBMC-derived macrophages were obtained using the same procedure as used for DC culture (section 2.3.2), except that 10% human serum was used and no cytokines were added (Swetman et al., 2002).

2.3.7 Flow cytometry

2.3.7.1 DC phenotypic analysis

DC phenotypic analysis was performed on day 8. DCs were incubated in Hanks's buffered saline solution (HBSS, Gibco) with 10% normal rabbit serum (RS) for 15 min to prevent non-specific binding. Cells were incubated with the relevant primary MAb for 45 min on ice, washed 3 times, and then incubated with fluoresceinated rabbit anti-mouse immunoglobulin (1:20 in HBSS/10%RS) for 45 min on ice. Cells were washed, fixed in 3.7% formaldehyde and data acquired on a FACScan flow cytometer (Becton Dickinson). Control samples included cells alone and an isotype control. Data were analyzed using CellQuest software. To analyse the effect on surface phenotype expression by SSL proteins, 10^5 cells were pre-incubated for 24 h in CM with 4.16 μ M of SSL7 or SSL9 or with peptidoglycan (PG, 5 μ g/ml, , from *S. aureus*, Sigma) or purified LPS (100 ng/ml, *Salmonella Minnesota*, Sigma) in 96 well U-bottomed plates at 37 °C. Cells were washed and stained with the relevant MAbs and examined on a FACScan flow cytometer as discussed above.

2.3.7.2 Binding and uptake of FITC labelled SSLs by human cells

Human peripheral blood mononuclear cells (PBMC) were generated from fresh whole blood samples obtained from healthy volunteers as described above. Binding assays were performed in U-bottomed 96-well microtiter plates by incubating 10^6 cells/well in CM with various concentrations of SSL-FITC (0.05-1.25 μ M) for 1 h at 4°C or 37°C. In some

experiments, 8 μ M of unlabelled SSL was added to the cells together with the labelled protein. After incubation, cells were washed 3 times by centrifugation, fixed in 2% formaldehyde and examined by flow cytometry.

2.3.7.3 *PBMC phenotypic analysis*

In some experiments, cells were additionally stained for various surface markers after SSL uptake. Cells were incubated with the relevant MAb markers, CD2, CD3, CD14, and CD19 for 30 min at 4°C, washed, and then incubated in 1:25-diluted phycoerythrin-conjugated goat anti-mouse immunoglobulin (PE, Jackson ImmunoResearch) for 30 min at 4°C. Cells were washed, fixed in 2% formaldehyde and examined using a FACScan flow cytometer.

2.3.7.4 *Dendritic cell uptake*

Dendritic cells (1×10^6 /ml) were incubated for 60mins in U-bottomed 96-well microtiter plates at 37°C with either SSL7-FITC or SSL9-FITC in CM. The cells then washed 3 times, fixed in 2% formaldehyde and examined by flow cytometry

2.3.7.5 *Endocytosis assay*

DCs (10^5) were incubated in CM with or without 4.16 μ M of SSL7 or SSL9 for various times (1 or 18 hours) in 96 well U-bottomed plates at 37 °C. Different concentrations (1, 3, 10, and 30 μ g/ml) of FITC-dextran (40,000 MW) were incubated with the cells. After 1 hour of incubation at 37°C, cells were washed in ice cold HBSS containing 0.1% azide to stop further endocytosis, fixed with 3.7% formaldehyde, and analysed by flow cytometry.

The uptake of dextran is expressed as mean fluorescent intensity. For each sample at least 5000 events gated on DCs were analysed.

2.3.7.6 *Cell line binding assay*

Different cell lines (Table 2.1) were cultured for 60 min at 37°C in U-bottomed 96-well microtiter plates with either SSL7-FITC or SSL9-FITC. The cells were washed 3 times, fixed in 2% formaldehyde and examined by flow cytometry.

2.3.8 Confocal microscopy

10⁵ cells were seeded on 32mm coverslips coated (for DC only) overnight at 4°C with 10µg/ml fibronectin (FN, Sigma) in HBSS (Gibco). After 2 h at 37°C in CM, cells were incubated with SSL-FITC (1.25 µM) and/or Texas Red-dextran (1mg/ml, Molecular Probes) for 1 h at 37°C in CM. right). Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI). The coverslips were then washed 3 times in cold HBSS and fixed in 2% paraformaldehyde. The slides were examined on a Bio-Rad confocal microscope. Images were acquired from 0.5-µm optical sections of individual cells.

2.3.9 SSL-specific antibody detection

Human serum was collected from heparinised blood of healthy volunteers. ELISA microassay plates were coated for 24 h with 0.04 μ M of SSL proteins dissolved in sodium carbonate buffer 0.1 M (pH 9.5) at 4°C (100 μ l/well). After three successive washes with HBSS containing 0.1% Tween 20, blocking was performed with 1% skimmed milk in HBSS for 1 h at 37°C. The plates were washed again three times, as before, and the test sera diluted at 1:2000 in HBSS containing 0.1% Tween 20 were incubated with SSL antigen for 1 h at 37°C (preliminary studies showed that this dilution of antisera gave no background staining for any serum tested in control wells). After three additional washes, the remaining bound antibodies were incubated for 1 h at 37°C with alkaline phosphates - conjugated goat anti-human antibodies diluted at 1:1000 in HBSS with 0.1% Tween 20. Excess conjugate was removed by washing as above and a colorimetric reaction was carried out by addition of the chromogen OPD (o-Phenylenediamine dihydrochloride) for 15-20 minutes. The plates were read at 405 nm to detect the optical density (OD) readings. A control well containing no serum was used to detect the background.

Competitive ELISA was performed by mixing (1:2000 final dilution) the sera with differing concentrations of SSL or a control bacterial protein Embp32 (0.08, 0.17, 0.33, and 0.42 μ M), and then testing binding on SSL-coated plates as above.

2.4 Statistical analysis

All experiments were carried out at least twice. Where appropriate, groups were compared using a 2-tailed Student's *t* test (using Microsoft Excel). The level of significance was <0.05.

2.5 Identification of SSL receptors

2.5.1 Purification of SSL receptors using SSL linked to Ni NTA agarose

Recombinant SSL proteins were expressed as discussed previously (section 2.1.1.2). Ni-NTA agarose was saturated with rSSL (SSL7 or SSL9) proteins by incubating 4ml of induced bacteria lysate with 1ml of Ni-NTA resin for 1 hour at 4°C. Afterward the resin (Ni-NTA-rSSLs) was packed into a column and washed with B-PER and 10 volumes of PBS. Two Ni-NTA-rSSL columns were used for each SSL protein (SSL7 or SSL9). In addition a blank column containing Ni NTA agarose without rSSL was used as a control to evaluate nonspecific binding. The cell line (1×10^9 cells of WMPT 3.3) suspended in B-PER buffer was sonicated (IKA-WERKE) on ice for 10-15 minutes, and the sonicate was centrifuged at 5000 rpm for 30 minutes.

This will remove debris and the receptors will be soluble in the supernatant. The localization of SSL receptors in the detergent-insoluble cell fractions that were removed by centrifugation is also possible. The efficiency of solubilisation could be checked by Western blotting of the supernatant and pellet, to detect the extent of solubilisation of known surface expressed receptors by specific antibody. In same manner, incubation of the blotting membrane with SSL proteins, followed by anti-SSL or anti-histidine tag and the detection antibody will show if the receptor is detectable by this methodology. This approach was not used initially, however, because it was thought less likely that the integrity of the receptor and its ability to bind SSLs would survive the denaturation steps required for Western blot.

Sufficient amount of cell lysate was allowed to bind to the columns at room temperature for 1 hour. The columns were then washed with 10 column volumes of PBS. Finally, bound proteins that interact with recombinant SSLs were eluted from each column using 8M urea (0.1M sodium phosphate pH 8), whereas SSL proteins were eluted from the Ni-NTA columns by decreasing the pH (4.5) of 8M urea (0.1M sodium phosphate pH 4.5). The material which eluted from the SSLs affinity-column was collected as 1 ml fractions, concentrated and separated by 10% SDS-PAGE.

2.5.2 Purification of SSL receptors using rSSL linked to NHS-activated Sepharose

NHS-activated SepharoseTM High Performance columns (Amersham, UK) were washed with 6 column (HiTrap 1ml) volumes of ice cold 1 mM HCl. Immediately after this columns were coupled overnight with 1ml of recombinant SSL protein (2 mg/ml of rSSL7 or rSSL9) at 4°C. Afterwards, the column's free-active groups were deactivated by washing with 0.5M ethanolamine, 0.5M NaCl (pH 8.3). The columns were then washed with 0.1M acetate, 0.5M NaCl (pH 4). A blank column was deactivated and used as control to evaluate nonspecific binding to the column.

Total cell lysate of WMPT 3.3 cell line was allowed to bind to the respective columns (NHS-rSSL7 or NHS-rSSL9) or blank NHS column at room temperature for 1 hour, the columns were then washed with 10 ml volumes of PBS. Finally, bound proteins that interact with recombinant SSLs were eluted from each column using 0.1M glycine (pH 2.1). Eluted cell proteins purified on SSL coupled affinity-columns were collected as 1 ml fractions concentrated (using Microcon centrifugation filter tube, 10 kDa) and separated on 10% SDS-PAGE. High salt buffer (KCl) was used in an attempt to elute the cellular

proteins that non-specifically bound to SSL proteins. Affinity columns were washed in a stepwise manner with 3 ml of PBS containing 0, 0.5, 1 and 2 M additional KCl, followed by 0.1M glycine (pH 2.1). All fractions were collected and separated on 10% SDS-PAGE.

2.6 Proteomic analysis

2.6.1 Generating peptides by trypsin digestion of proteins

Proteins of interest were excised from gels and transferred to a clean 0.5 ml Eppendorf tube. Gel slices were washed three times for 15 minutes per wash in a rotary mixer with 400µl of 50% acetonitrile / 25mM ammonium bicarbonate (pH 8) to remove excess stain, Gels slices were dehydrated in 100% acetonitrile for 5 minutes, and completely dried in a Speed-Vac (Savant) for 20-30 minutes. Trypsin (Promega) was diluted to a concentration of 15 µg/ml in 25mM ammonium bicarbonate. Each gel slice was soaked with 15-20 µl of cold trypsin, and incubated for 16-24 hours at 37°C.

2.6.2 Peptide extraction and ZipTip C18 purification

Peptides generated by in-gel digestion (section 2.6.1) were extracted from gel slices twice with tri-fluoroacetic acid (TFA). 50µl of 0.1% of TFA was added to each slice and incubated for 60 minutes with shaking, and then the supernatant were collected in a new tube. Extracts were completely dried (1 hr) in a Speed-Vac. Peptides were resuspended in 10 µl of 0.1% TFA. ZipTip C₁₈ micro-columns (Millipore) were used to remove salts or organic contaminants. Each tip was conditioned with 10µl of 100% acetonitrile, and three washes of 10µl of 0.1% TFA. Contaminants were removed by washing the ZipTip three times with 10µl of 0.1% TFA.

2.6.3 Peptide fingerprinting by MALDI-TOF MS

Peptide-containing samples were eluted from the ZipTips using 5 µl α -cyano-4-hydroxycinnamic acid (CHCA) matrix, made up in 0.1% TFA, 50% acetonitrile, and were spotted in 0.5 µl droplets onto a MALDI-TOF MS sample plate in duplicate. A calibration mix containing angiotensin and ACTH peptide standards (Perseptive Biosystems) were spotted in corresponding wells on the plate, to enable external calibration of the samples. The spots were allowed to dry at room temperature before being loaded into a Voyager DE Pro MALDI-TOF mass spectrometer. Peptide fingerprints were generated by ionisation of the sample/matrix complex using a 327nm Nitrogen laser with delayed extraction. Mass to charge ratios were calculated based on the time of flight detection of ions.

MALDI-TOF MS was also done by Dr Steven Howell, division of protein structure, National Institute for Medical Research, London.

2.6.4 Identification of proteins by database searching

Proteins were identified using Protein Prospector v3.5 software. Briefly, representative isotopic peak masses obtained by MALDI-TOF MS analysis were used to search the NCBI database using Protein Prospector v3.5 software.

CHAPTER III

Cellular tropism of staphylococcal superantigen like proteins

3. Cellular tropism of staphylococcal superantigen like proteins

3.1 Introduction

The presence of eleven members of the SSL protein family in all *S. aureus* strains tested so far, suggested that these proteins have important non-redundant biological functions as agents of host/pathogen interactions. In contrast to classical superantigens, SSLs do not show the main properties of superantigens such as polyclonal T cell activation, pyrogenicity, or enhancement of endotoxin shock. However, SSLs may function to distract the host's immune system, but do so via entirely different molecular mechanisms and participate in the pathology of staphylococcal diseases.

Identification of the cellular binding partner for the SSL proteins would be a milestone for understanding and determination of the function(s) of these secreted proteins. Moreover, this would also provide information on the involvement of these proteins in the pathogenicity of *S. aureus*. A first step in characterising the SSL function and its cellular receptors is to identify whether SSL interacts selectively with cells of the immune system. Therefore, the main objective of this chapter was to investigate the interaction of SSL7, and its close homologue SSL9, with different cells of the immune system.

In an attempt to design a strategy to assess whether these proteins have the ability to bind to PBMCs, the recombinant SSL proteins were expressed, purified and directly labeled with FITC. FITC labeled SSLs were then allowed to interact with various immune cells and analysed by the mean of flow cytometry.

3.2 Results

3.2.1 Interaction of SSL proteins with PBMCs

Peripheral blood mononuclear cells (PBMC) (Fig. 3-1) were incubated with various concentrations of SSL7-FITC or SSL9-FITC for 1 h, and cell-associated fluorescence measured by flow cytometry. Both SSL7 (Fig. 3-2 A) and SSL9 (Fig. 3-2 B) stained a small proportion of PBMC at 37°C, but not at 4°C. The level of fluorescence was dose-dependent over the range tested (0 - 1.25 μ M). The mean percentage of cells stained with 1.25 μ M of SSL7 (9.8 ± 1.8 , range 7.1-12.2, $n=7$) and 1.25 μ M of SSL9 (10.9 ± 1.1 , range 9.4-12.6, $n=5$) was very similar. Mean fluorescence also increased with time between 5 minutes and 120 minutes (Fig. 3-3) suggesting progressive uptake of SSL protein by the cells.

In order to determine whether the interaction between SSL protein and PBMC was specific, competitive inhibition of SSL-FITC cell labelling by unlabelled SSL was investigated (Fig. 3-4). Excess unlabelled SSL7 was able to completely block uptake of SSL7-FITC. In contrast, neither SSL9, nor an unrelated bacterial protein also carrying a polyhistidine tag (Embp32) had any effect on the SSL7-FITC signal. Conversely, only unlabelled SSL9, but not SSL7 or Embp32, blocked uptake of SSL9-FITC. Interaction between SSL proteins and the PBMC therefore occurs via a specific receptor, and is not mediated by the histidine tag on these proteins. Furthermore, SSL7 and SSL9 use different receptors, or different sites within one receptor.

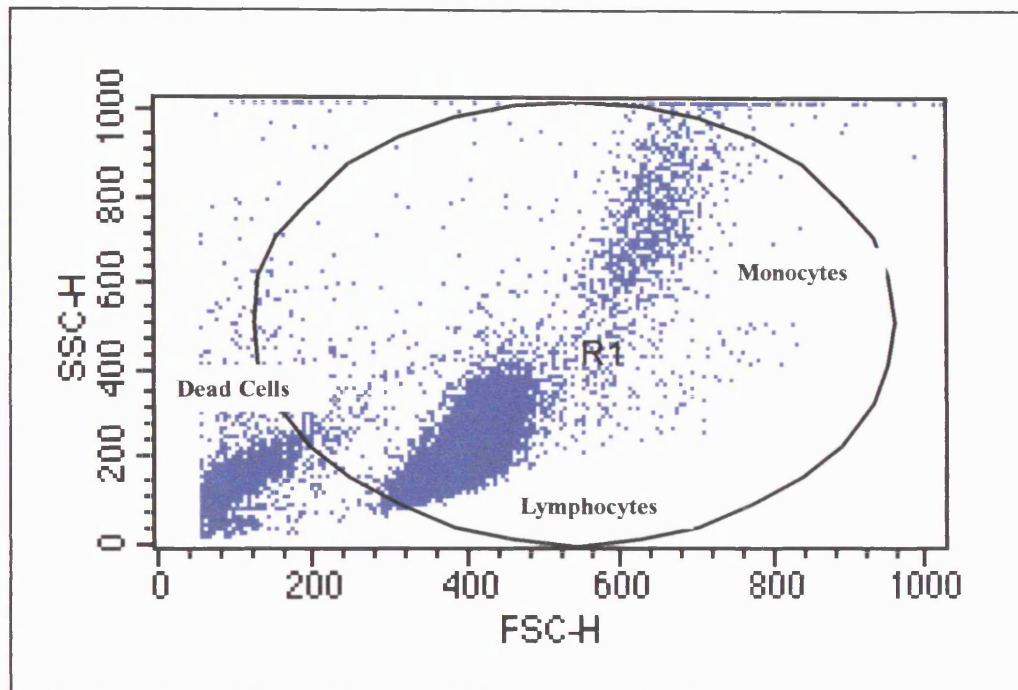
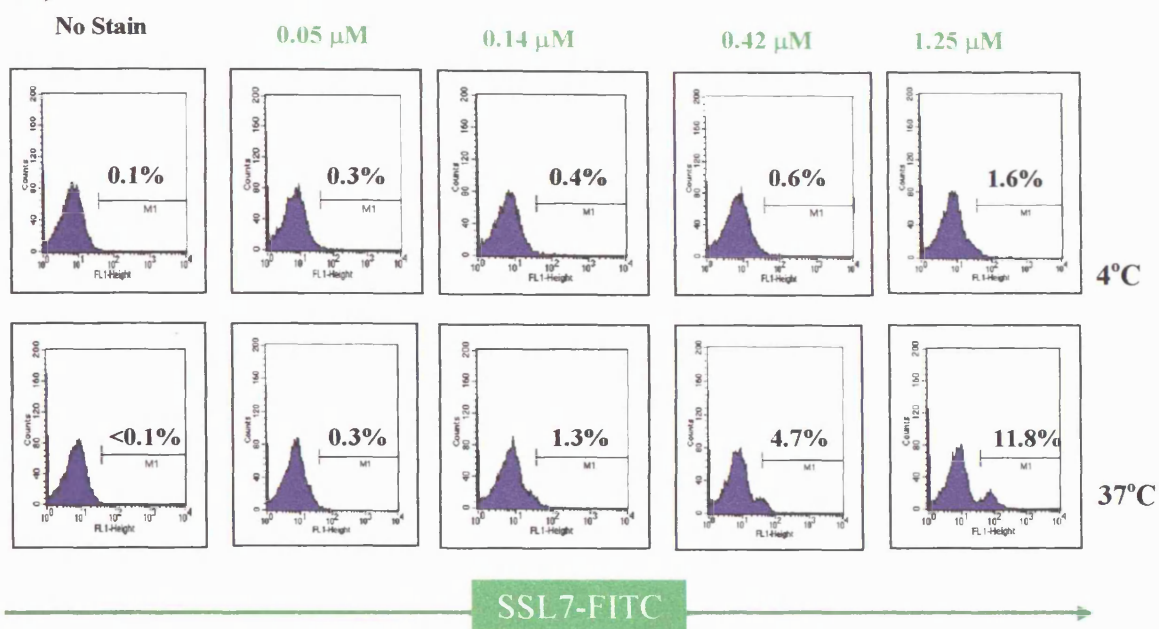


Figure 3-1 Flow cytometry: forward scattered (FCS) / side-scatter (SSC) dot plot of PBMCs.

Cells in the circular gate were isolated from normal individuals and used for SSL binding assay by FACScan.

A)



B)

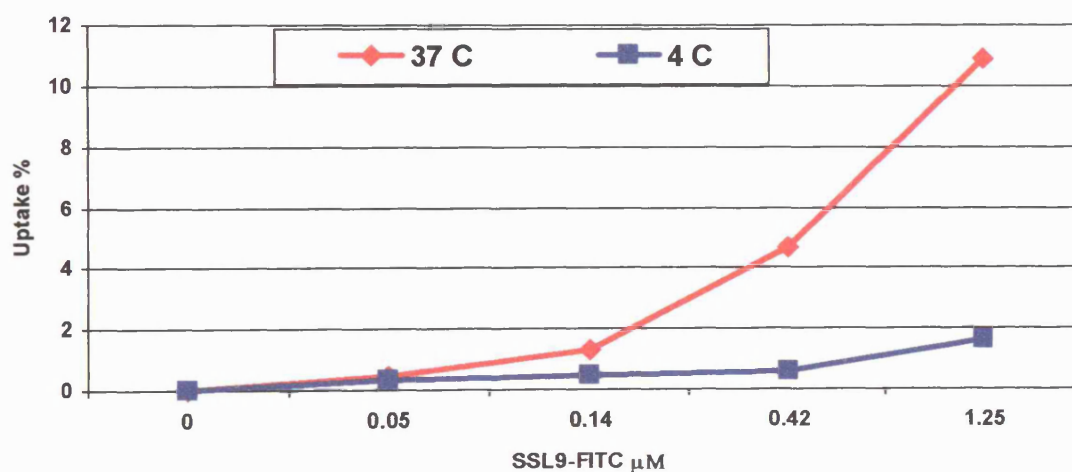


Figure 3-2 Dose and temperature dependence of SSL uptake by PBMCs.

PBMCs were incubated for 60 minutes with varying concentrations of SSL7-FITC (A) or SSL9-FITC (B) at 37°C or 4°C, and the binding was measured by flow cytometry. The numbers show the percentage of uptake of SSL proteins by PBMCs. These graphs are representative of 10 separate experiments carried out with PBMCs from healthy donors.

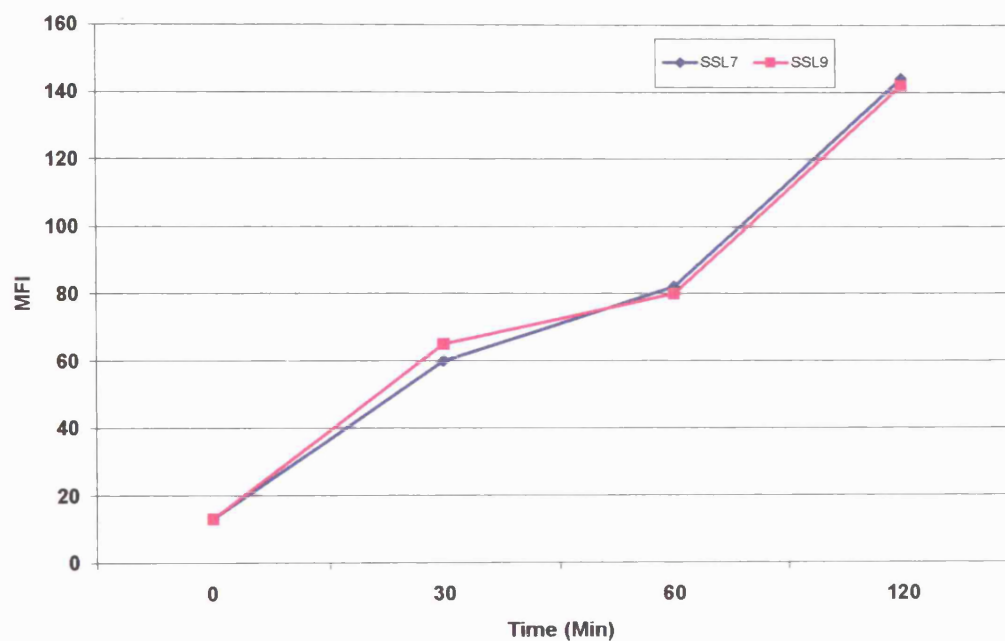


Figure 3-3. Time dependence of SSL uptake by PBMCs.

PBMCs were incubated for various times with 1.25 μM of SSL7-FITC or SSL9-FITC at 37°C, and the uptake was measured by flow cytometry. This graph is representative of 3 separate experiments carried out with PBMCs from healthy donors.

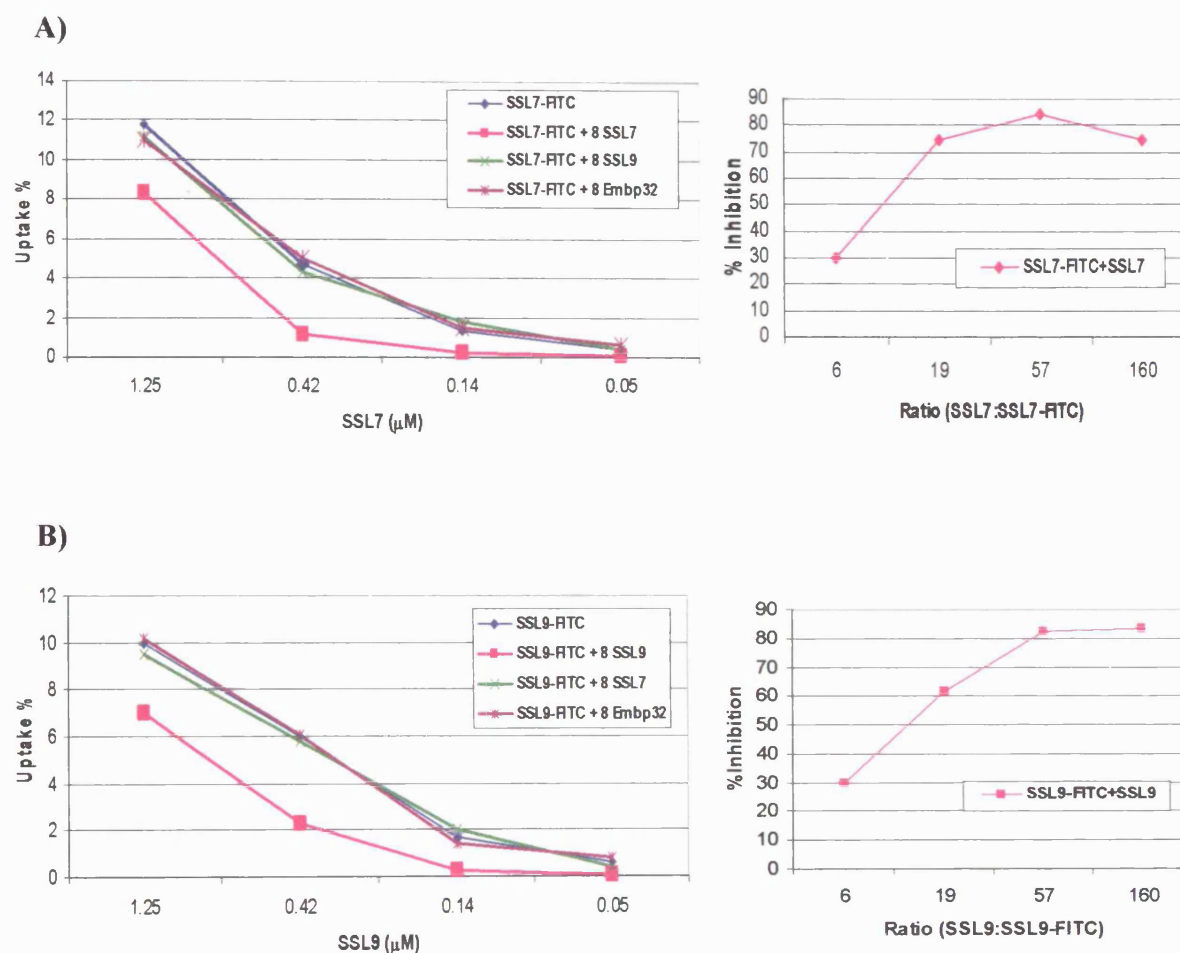


Figure 3-4. Inhibition of binding of SSL-FITC to PBMCs by unlabelled SSL protein.

Different concentrations of SSL7-FITC (A) or SSL9-FITC (B) were incubated with PBMCs with or without 8 μM of unlabelled SSL7, SSL9 or Embp32 protein, and the uptake was measured by flow cytometry. Results shown are from one representative experiment of three. The right panels show the percentage of inhibition observed at different ratios of unlabelled to labelled SSL7 and SSL9.

3.2.2 Characterisation of PBMC sub-population targeted by SSL proteins

The PBMC sub-populations which are the targets for SSL7 and SSL9 were further characterised by immunophenotyping, using monoclonal antibodies to the major surface markers CD2, CD3, CD14, and CD19. Both SSL7 and SSL9 (Fig. 3-5 and 3-6) were taken up by all CD14 positive cells, and by a population of CD2-low cells, a phenotype consistent with that of peripheral blood monocytes (Crawford et al., 1999). Neither SSL7 nor SSL9 showed any interaction with CD3 positive T cells. Interestingly, SSL7-FITC but not SSL9-FITC stained a subpopulation of CD19 B cells, providing further evidence that the receptor for these two SSLs is distinct.

In order to confirm that the binding of SSLs to PBMCs was not affected by the presence of the histidine tag, SSL7 was subcloned into pGEX-4T-1, expressed, and purified, and the GST tag was then removed by cleavage with thrombin. FITC-labelled SSL7 lacking a tag showed binding characteristics very similar to those of the histidine-tagged version (Fig. 3-7). In particular, both versions of SSL7 bound to only a subpopulation of HLA-DR-expressing cells, suggesting that the proteins did not bind directly to this receptor.



Figure 3-5. Immunophenotype of PBMC that interact with SSL7 protein. PBMCs were stained for various cell surface markers after incubation with different concentrations of SSL7-FITC for 60 minutes at 37°C. This graph is representative of 3 separate experiments.

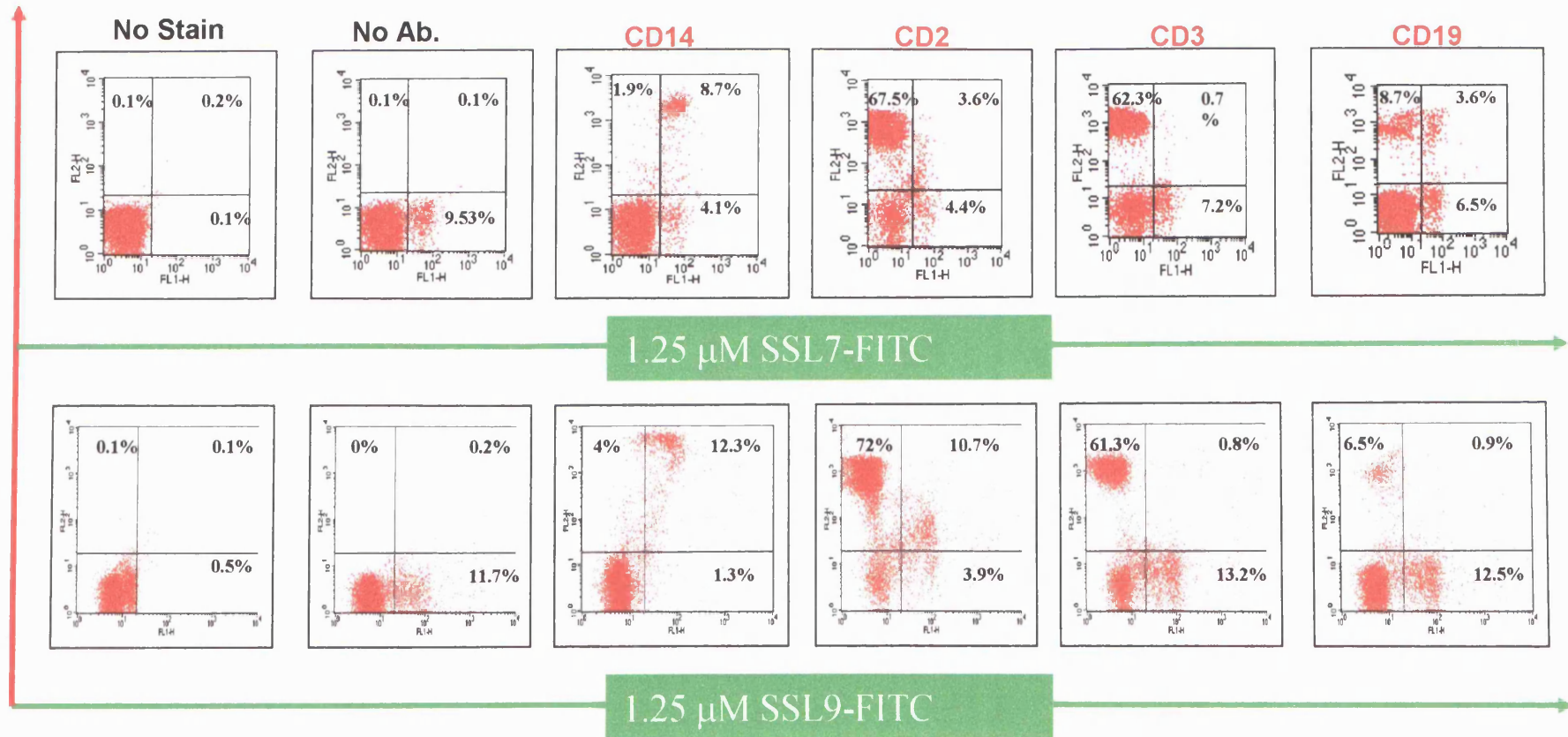


Figure 3-6 Immunophenotype of PBMC that interact with SSL proteins. PBMCs were stained for various cell surface markers after incubation with 1.25 μ M SSL7-FITC or SSL9-FITC for 60 minutes at 37°C. This graph is representative of 5 separate experiments.

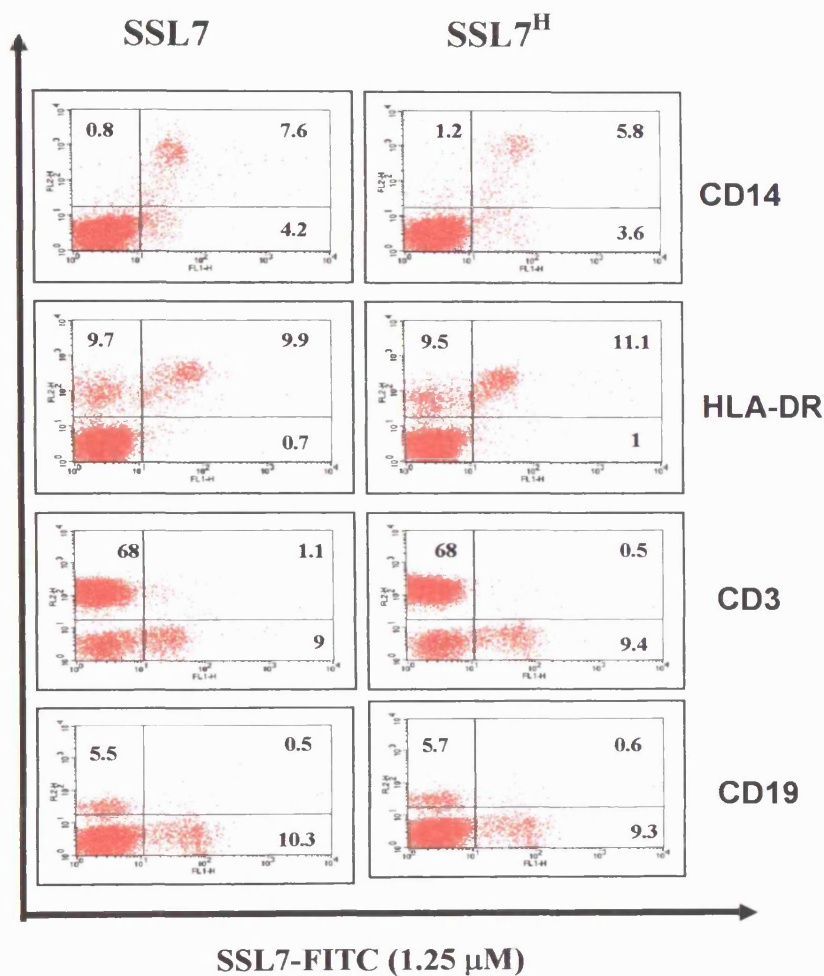


Figure 3-7. Comparison of staining observed with SSL7-FITC with the histidine tag (SSL7^H) (graphs on the right) and without the histidine tag (graphs on the left). Immunophenotype of PBMC that interact with SSL proteins. PBMCs were stained for various cell surface markers after incubation with 1.25 μ M SSL7-FITC for 60 minutes at 37°C, and the uptake was measured by flow cytometry

3.2.3 Uptake of SSL proteins by dendritic cells

Peripheral blood monocytes were cultured *in vitro* in the presence of GM-CSF and IL-4, in order to drive their differentiation into myeloid dendritic cells (Sallusto and Lanzavecchia, 1994). After depletion of residual lymphocytes, the population obtained after seven days culture consisted of >90% CD1a⁺ HLA-DR^{high} CD14^{low} dendritic cells (Fig. 3-8). These cells were incubated for 60mins at 37°C with either SSL7-FITC or SSL9-FITC and examined by flow cytometry and confocal microscopy (Fig. 3-9 and 3-10). Dendritic cells stained uniformly strongly positive for both SSL7 and SSL9. Confocal microscopy confirmed that fluorescence was predominantly due to intracellular uptake of SSL, rather than surface staining. Both SSL7 and SSL9 were concentrated in small vesicular structures, localised particularly to the perinuclear region of the cell.

In order to characterise the nature of these vesicles further, dendritic cells were cultured in the presence of SSL7 or SSL9-FITC and Texas Red dextran (Fig. 3-11), which is avidly taken up by DC via mannose receptors (Sallusto et al., 1995). Texas Red dextran strongly labelled a large number of intracellular vesicles throughout the DC cytoplasm. SSL distribution and dextran distribution partially overlapped, with some intracellular vesicles clearly containing both markers. However, SSL positive dextran negative vesicles were also observed. In a small proportion of cells, vesicles containing SSL9 appeared to aggregate, to generate very large vesicles, which contained high concentrations of both SSL and dextran (Fig. 3-11). The very large vesicles observed (which were never seen in the presence of dextran alone) presumably resulted from fusion of many SSL containing

vesicles, and may have been driven by intramolecular interactions between SSL molecules. Similar vesicle distortion is observed in the presence of excess invariant chain, again driven by multiple interactions between invariant chain molecules (Romagnoli et al., 1993).

In order to determine whether uptake of SSLs was a generalised feature of endocytic cells, peripheral blood monocytes were differentiated into macrophages, via culture in human serum, without added cytokines. Under these culture conditions, the cells develop a completely different phenotype (CD1a-,HLA-DR-,CD14^{high}) and morphology (lack of dendrite formation) (Swetman et al., 2002). Macrophages, like DC efficiently endocytosed Texas Red dextran, but showed no uptake of either SSL7 or SSL9 (Fig. 3-12 and 3-13).

Other fluorescent dye such as Alexa Fluor could be used as an alternative to FITC for SSL proteins labelling to rule out any possibility of FITC quenching by the lower pH of the endosomal acidic vesicles in macrophages.

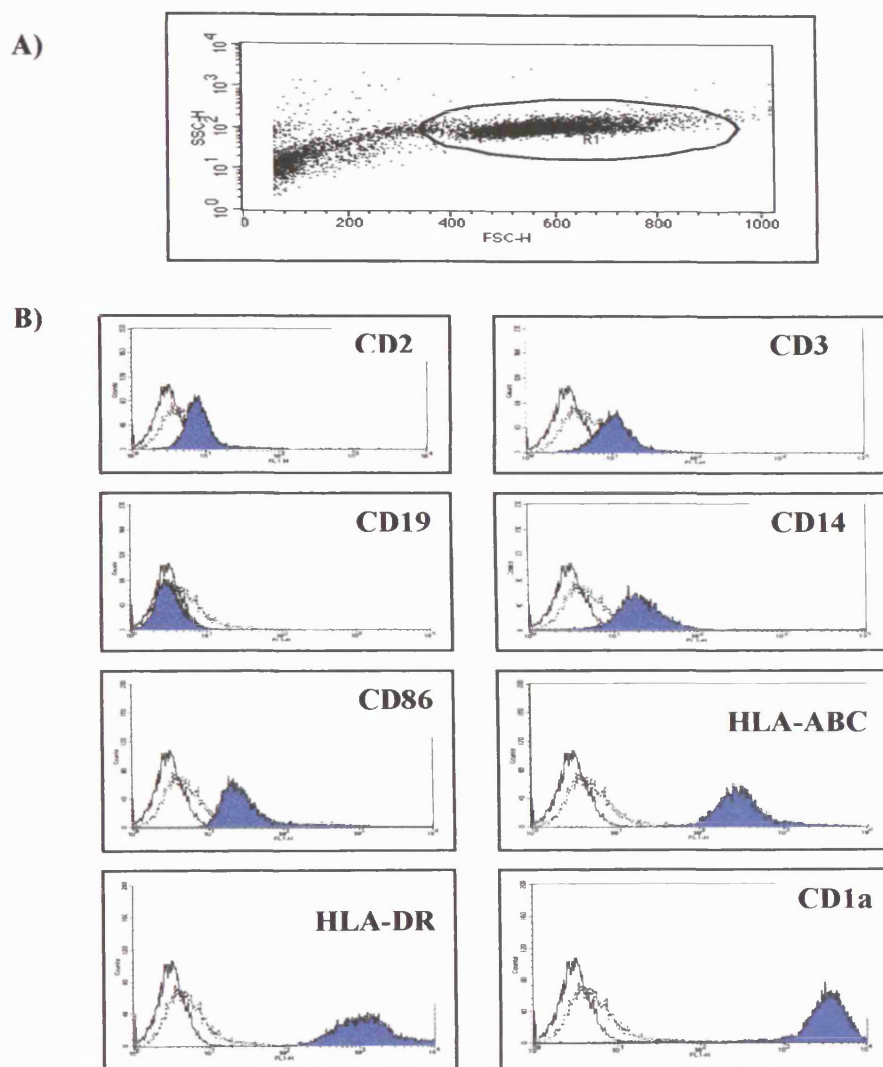


Figure 3-8. Phenotypic analysis of DC.

(A) Forward scatter/side scatter dot plot of DC. Cells in the elliptical gate have characteristic (size/granularity) of DC. (B) Day 7 DCs were isolated, incubated with MAbs and analysed by FACScan. The indicated markers are shown by the blue histograms, whereas cells either alone (thick line) or stained (dotted line) with relevant isotype control MAb are indicated by the open line histograms. Results shown are from one donor and are representative of similar data obtained from experiments carried out with DC from three different donors.

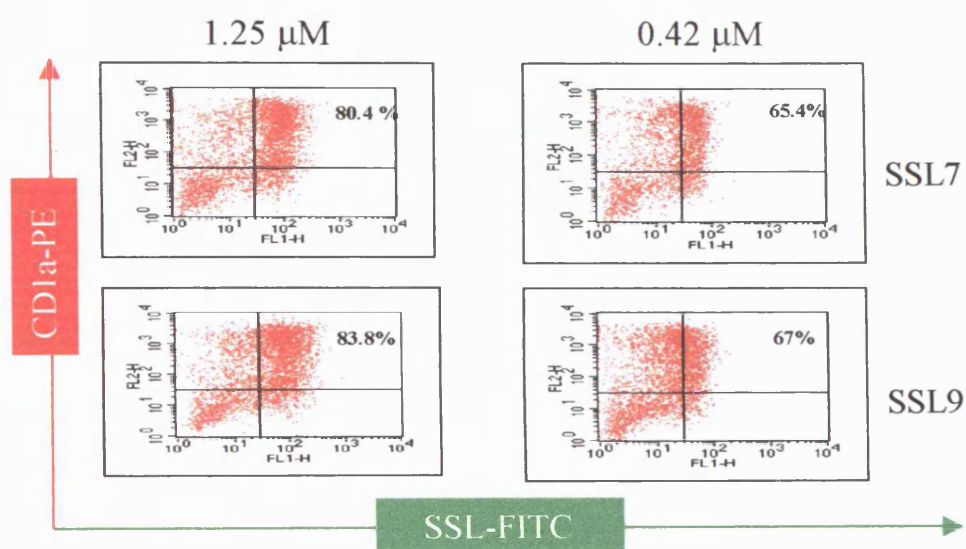


Figure 3-9. SSL proteins binding to PBMC-derived dendritic cells (DC).

CD1a⁺ DCs (unpurified) derived from CD14⁺ cells were incubated with different concentrations of SSLs conjugated with FITC. The cells were fixed and analysed by flow cytometry, measuring SSL as FL1 (horizontal) and CD1a by FL2 (vertical). The numbers show the percentage of SSL positive cells that were also CD1a positive. This figure is representative of a set of 3 separate experiments carried out with DCs from healthy donors.

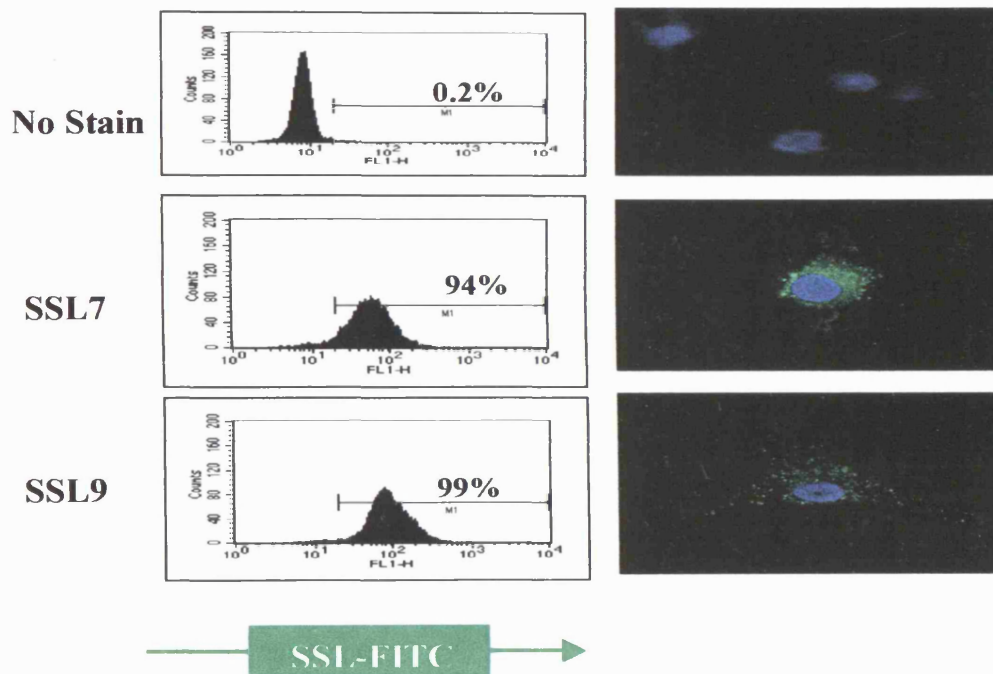


Figure 3-10. SSL proteins binding to PBMC-derived dendritic cells (DC).

Purified DCs derived from CD14⁺ cells were incubated with 1.25 μ M of SSL conjugated with FITC. Binding was analysed by flow cytometry (left panel) and confocal microscopy (right panel). Nuclei are stained with DAPI and shown in blue. This figure is representative of a set of 3 separate experiments carried out with DCs from healthy donors.

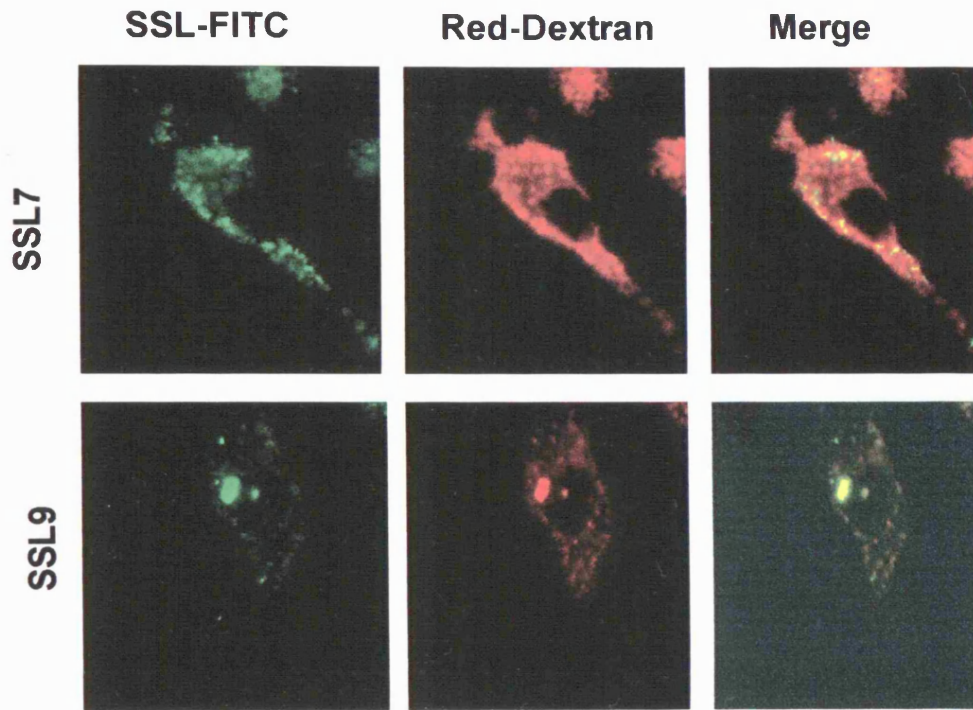


Figure 3-11. Intracellular localization of SSL proteins in human PBMC-derived dendritic cells (DC).

Dendritic cells were cultured in the presence of 1.25 μ M SSL7 or SSL9-FITC with or without 1mg/ml Texas Red dextran for 60 minutes, 37°C. Cells were analysed by means of confocal microscopy. The images shown are representative of 6 separate experiments.

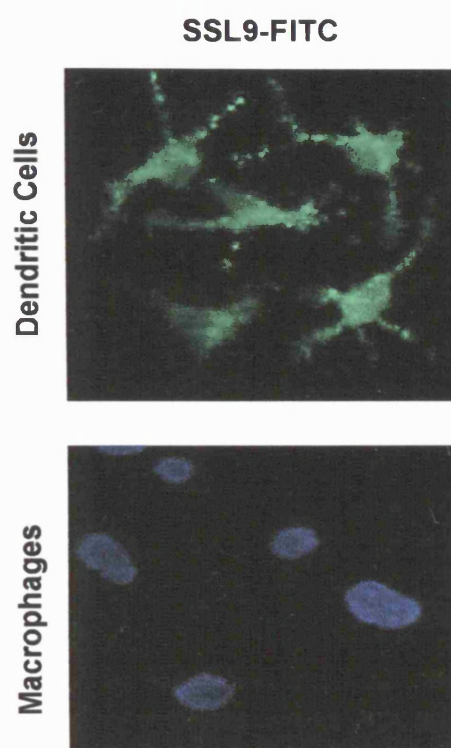


Figure 3-12. Intracellular uptake of SSL proteins by PBMC-derived dendritic cells (DC) and PBMC-derived macrophages.

Cells were incubated with 1.25 μ M SSL9-FITC for 60 minutes, 37°C. The images shown are representative of 3 separate experiments.

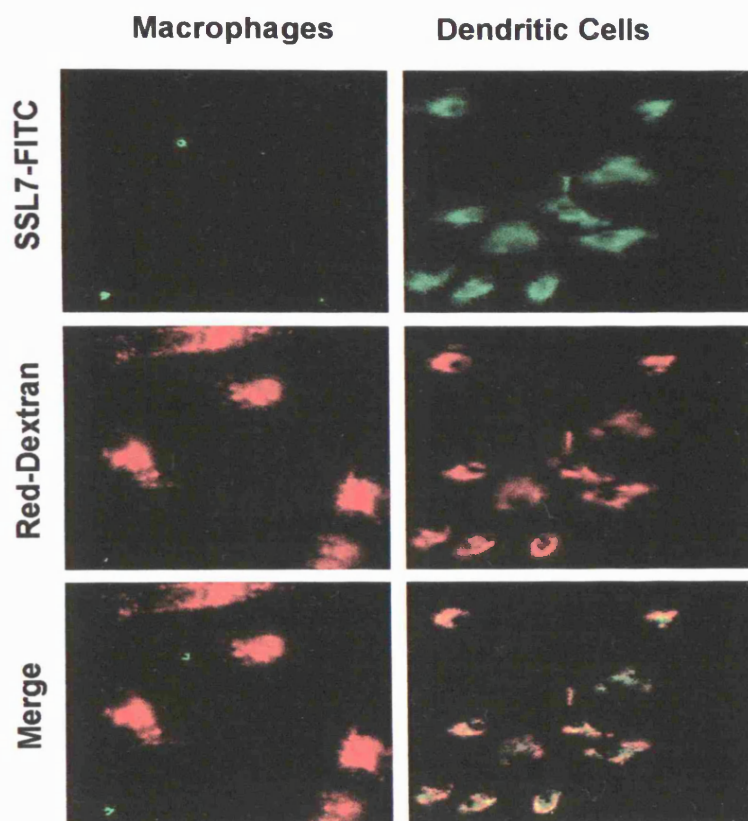


Figure 3-13. Intracellular uptake of SSLs by PBMC-derived dendritic cells and PBMC-derived macrophages.

Cells were incubated with 1.25 μ M SSL7-FITC and 1 mg of Texas Red dextran per ml for 60 min at 37°C. The results are representative of the results of three separate experiments

3.3 Discussion

The characteristic features of the interaction between SSL7 and SSL9, and PBMC are specificity, temperature dependence and cell selectivity. Specificity, indicative that the interaction is mediated by a cell surface receptor, is shown by the demonstration that unlabelled SSL blocks uptake of SSL-FITC. This competition is observed for both SSL7 and SSL9. The lack of reciprocal inhibition between SSL7 and SSL9 suggest that these two molecules have different binding partners on the cell surface, although we cannot rule out the possibility that they bind to different sites on the same molecule. Due to limitations in the concentration of labelled SSL proteins, we could not determine the amount of SSL proteins that were able to saturate the putative receptors. Since it was impossible to measure binding in the absence of uptake, true measurements of affinity could not be obtained. The concentrations required to obtain measurable uptake, however, were in the order of 0.1 micromolar, suggesting that the affinity of interaction with any putative receptor is relatively low. This is a characteristic of many classical superantigens (Labrecque et al., 1993), and also of many receptors of the innate immune system (e.g. mannose receptors, (East and Isacke, 2002; Sallusto et al., 1995). Inhibition of SSL proteins uptake by cytochalasinD or colchicine, inhibitors for microfilaments or microtubules respectively, would be useful approaches to understand the cellular uptake mechanism for this family of proteins.

The temperature and time dependence of SSL interaction are suggestive of receptor mediated uptake rather than simple binding to the cell surface, and this was confirmed by the confocal microscopy studies discussed further below. However, a small amount of surface binding can be detected at 37°C, but not 4°C, using indirect labelling of intact

cells with an antibody against the histidine tag (not shown). The interaction of SSL with the receptor, as well as its subsequent uptake, is therefore temperature-dependent.

The third characteristic of SSLs observed in these cellular studies with PBMC was the highly selective nature of the target population with which interaction could be detected. In *ex vivo* PBMC, the major target population is the monocyte, characterised by high expression of CD14. Essentially all monocytes were found to interact with both SSL7 and SSL9. In contrast, neither SSL7 nor SSL9 interacted with T cells, identified by expression of CD3 and high levels of CD2. Interestingly, SSL7, but not SSL9 also bound to a proportion of B cells (in the order of 30% although this varied significantly between individuals), providing further evidence that the receptor for these two molecules is distinct. The B cell sub-population which interacts with SSL7 could be CD5 cells as less than 30% of circulating B cell in adults are positive to this marker (Youinou et al., 1999), but this was not investigated further in this study. Since a very significant proportion of T cells and all human B cells also express class II MHC (e.g. HLA-DR) this result rules out a direct binding of SSL7 or SSL9 to these molecules, thus clearly distinguishing them from classical superantigens.

Monocytes express both class I and class II MHC molecules, and can act as antigen presenting cells for the activation of CD4 or CD8 T cells. However, the prototype antigen presenting cell, and the only cell type which can activate naïve T cells, is the dendritic cell. It was therefore of interest that both SSL7 and SSL9 were taken up efficiently by monocyte-derived DC. This cell type, which can be obtained by culture of PB monocytes with appropriate cytokines, provides a widely used model for myeloid dendritic cells.

In contrast, neither SSL7 nor SSL9 showed any tropism for macrophages, a cell type also produced by *in vitro* culture of monocytes, but which does not present antigen except when stimulated by appropriate cytokines, such as IFN γ . This result suggests that macrophages do not express receptors for SSLs proteins. Although macrophages may take up SSLs via nonspecific mechanisms (i.e., macropinocytosis), the concentrations of labelled protein used are likely to be too low to detect this uptake. Further experiments to see whether macrophages can be induced to express the SSL receptor following activation would be of interest.

Confocal studies on intracellular co-localization of SSL-FICT proteins would highlight the possible staphylococcal target strategy within DCs. Staining of endocytic vesicles such as class II MHC (MHC loading compartment), cathepsins (proteinases in Ag processing) or ER-specific markers BiP (GRP78) in antigen presenting cells (see section 5.2.5) may be of interest.

CHAPTER IV

**Functional studies of dendritic cells exposed to
Staphylococcal superantigen-like proteins**

4. Functional studies of dendritic cells exposed to SSL proteins

4.1 Introduction

In the previous chapter we demonstrated the interaction between two members (SSL7 and SSL9) of the SSL protein family and human immune cells. These proteins target themselves selectively to APCs such as monocytes and DC, leading to progressive uptake and internalisation by these cells. Dendritic cells have a key function in initiating and connecting both arms of the immune response. Therefore, we hypothesised that *S. aureus* may use SSL proteins as an evasion strategy to induce alterations in the functional properties of DC, such as DC maturation, uptake, and antigen presentation as well as DC-mediated T cell activation, preventing their normal function and thus moderating the response of the host to the bacterium. In this chapter, we test this hypothesis.

The other objective of this chapter was to determine the immunological response to the SSL proteins in the normal human population. Very little work has been carried out previously on T cell responses to *S. aureus*. Therefore, the proliferative and cytokine responses of T cells, as well as the antibody response against these proteins were examined to demonstrate the presence and type of T cell specific response toward this family of proteins, and the presence of specific SSL antibodies in this population of normal individuals.

4.2 Results

4.2.1 The effects of SSL proteins on DC viability, morphology and surface phenotype

Monocyte-derived DCs (MDDC) were derived from PB monocytes. They showed the characteristic phenotype of immature DC; low CD14, and high HLA-DR and high CD1a (see Fig 3-8). As shown previously (see section 3.2.3), fluoresceinated SSL7 and SSL9 labelled DC in purified cultures, containing less than 5% non-DC.

DCs were incubated with SSL7 or SSL9 (4.16 μ M) for either 1 or 18 hours at 37°C, and cell viability was assessed by trypan blue exclusion. Microscopic analysis revealed that SSL proteins were not cytotoxic as more than 95% of cells appeared viable. Untreated cells were predominantly non-adherent with few dendritic cell processes, typical of immature DC (Fig 4-1). Neither SSL7 nor SSL9 induced any noticeable morphological changes over the time period tested. In contrast DCs treated with the TLR4 bacterial ligand LPS (100 ng/ml) or the TLR2 ligand peptidoglycan (PG) (5 μ g/ml) became adherent and extended multiple, long dendritic processes (Fig 4-1).

The cell-surface expression of a panel of characteristic DC surface markers was analyzed by flow cytometry. Immature DCs were incubated with 4.16 μ M of SSL7 or SSL9, or LPS or PG and the surface phenotype of these DCs was analyzed after 18 h of culture. Neither SSL7 nor SSL9 (Fig 4-2) induced significant changes in any of the surface molecules measured. In contrast, DCs incubated with either LPS or PG up-regulated surface expression of HLA-DR, HLA-ABC, CD86, and CD54. Thus, in summary, exposure of

DCs to the SSLs protein did not induce DC maturation, nor indeed any obvious changes in DC surface phenotype, viability or morphology.

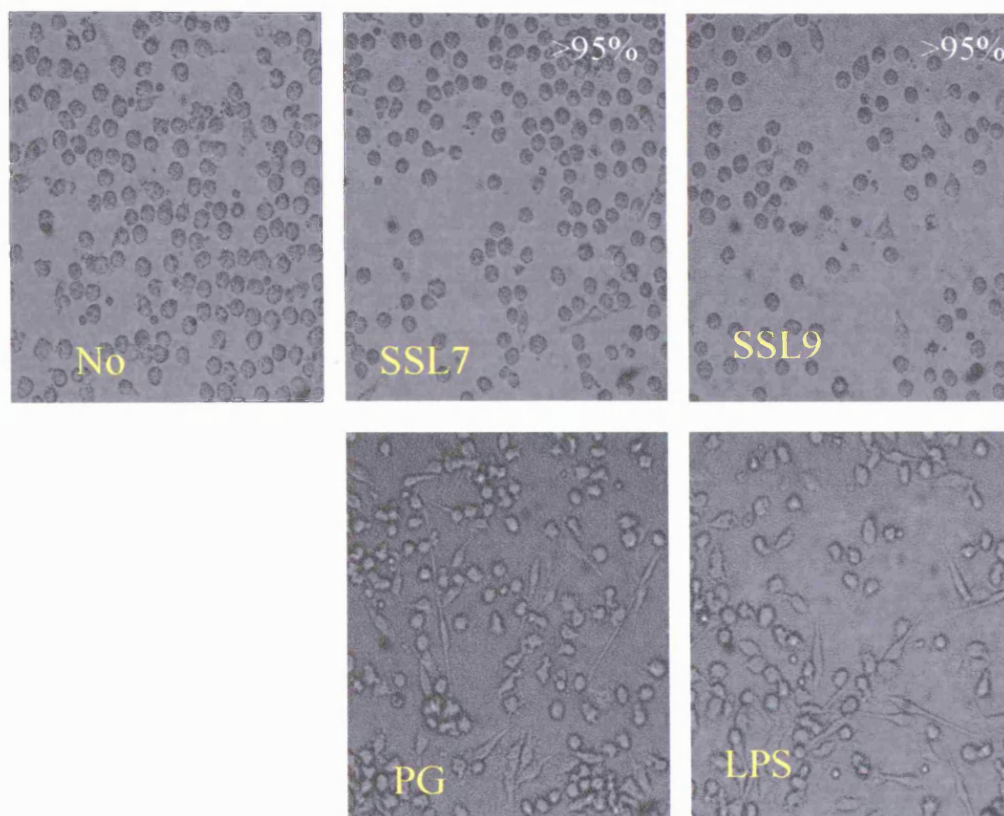


Figure 4-1 Morphology of DC treated with SSL proteins.

DC were cultured in the presence of 4.16 μ M SSL7 or SSL9 for 18 hours at 37°C. Lipopolysaccharide (LPS, 100 ng/ml) and peptidoglycan (PG, 5 μ g/ml) were used as positive controls. The numbers in the top right corner show the percentage of cells which were trypan blue negative. Representative of 3 experiments.

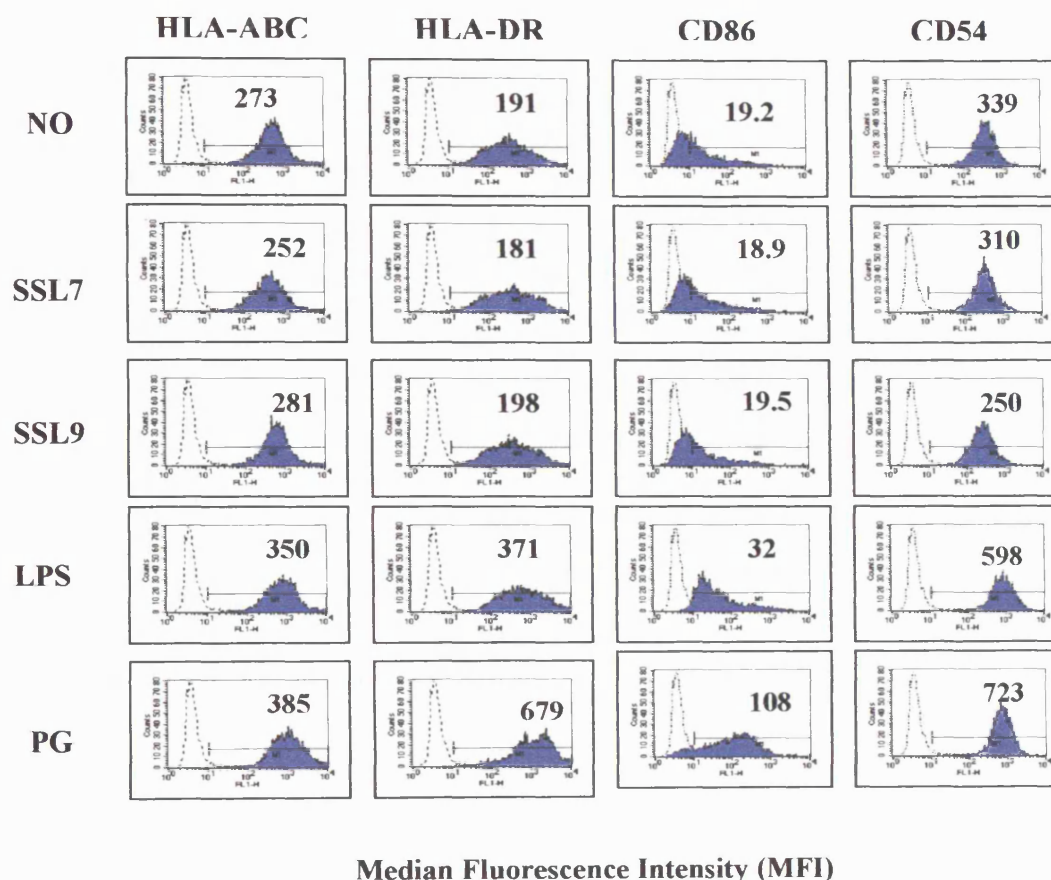


Figure 4-2. Phenotypic analysis of DC treated with SSL proteins.

Data are shown for expression of cell surface molecules on DC that had been treated for 18 h with 4.16 μ M SSL7 or SSL9. Lipopolysaccharide (LPS, 100 ng/ml) and peptidoglycan (PG, 5 μ g/ml) were used as positive controls. Expression of the indicated markers is shown by the solid histograms, whereas cells stained with control mAb are indicated by the open line histograms. The numbers on each histogram correspond to the median fluorescence intensity (MFI) of mAb staining. Results shown are from one donor and are representative of similar data obtained from experiments carried out with DC from four different donors.

4.2.2 The influence of SSLs on endocytosis

Fluorescein isothiocyanate-labelled dextran (FITC-Dx) is rapidly taken up by DCs via the mannose receptor (Sallusto et al., 1995). To determine whether SSL protein altered DCs antigen uptake function, DCs were treated with 4.16 μ M of SSL7 or SSL9 and incubated for 1 or 18 hours at 37°C. Different concentrations of FITC-Dx were added to the cell and incubated for a further hour at 37°C, and FITC-Dx uptake by the DC was then measured by flow cytometry. As shown in Fig 4-3, SSL treated DC showed rapid uptake of FITC-Dx, and neither protein had any effect on endocytic activity.

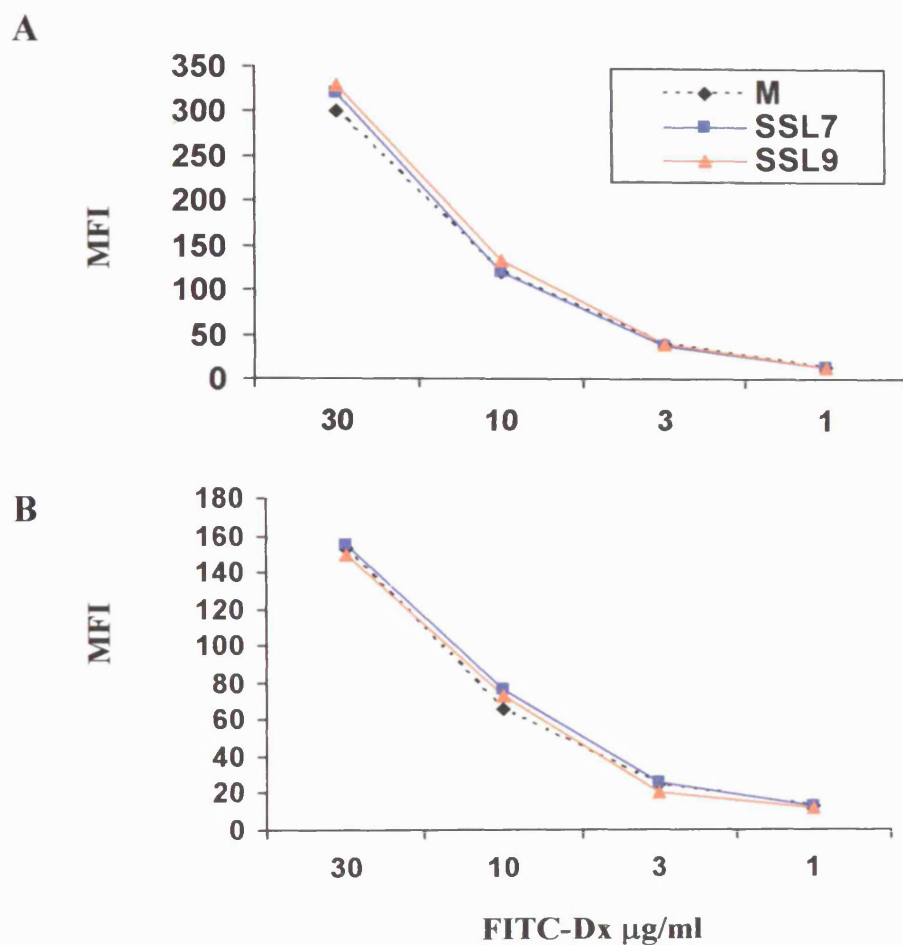


Figure 4-3. Endocytosis of FITC-Dx by DCs exposed to SSL proteins.

DCs were incubated for (A) 1 hour or (B) 18 h in presence or absence of SSL7 or SSL9 (4.16 μM). Excess proteins were removed by washing, and the cells were incubated with different concentrations of FITC-dextran (1, 3, 10, and 30 $\mu\text{g/ml}$). Excess dextran was removed by washing, and the cells were fixed. The total cell associated dextran was measured by flow cytometry, and expressed as mean fluorescent intensity for a minimum of 5000 DC. The results of one of three separate experiments are shown.

4.2.3 The influence of SSLs on T cell stimulatory capacity of DCs

To evaluate the effect of SSL proteins on the stimulatory capacity of DC in T cell proliferation, day 6 DCs were incubated for 18 hours with different concentrations of SSL proteins (0.42, 1.25 and 4.16 μ M). Residual T cells were depleted and the functional assays performed using fresh viable purified autologous or allogeneic T cells. The ability to induce secondary immune responses was unchanged. Figure 4-4A shows representative experiments eliciting recall responses to tuberculin (PPD). There was no statistical difference in the proliferative responses observed between any of the pre-incubated DC groups with SSL7 or SSL9 (37182 ± 2036 cpm and 36458 ± 3000 cpm, respectively) and the control (36458 ± 6151 cpm) after the 6 days co-culture period ($P > 0.05$).

The capacity of SSL-treated DCs to elicit primary T cell proliferation also was tested in an allogeneic mixed lymphocyte reaction (MLR). A similar result was observed (Fig 4-4B), the proliferation response against allogeneic T cell (25710 ± 1140 cpm) was unaffected by exposure to SSL7 or SSL9 (26149 ± 3674 and 25816 ± 3159 cpm, respectively, ($P > 0.05$). Therefore, SSL proteins have no effect on the ability to induce proliferation of allogeneic T cells. In general, the results of these experiments demonstrated that the antigen presentation capacity of DCs remains intact in the presence of these secreted proteins.

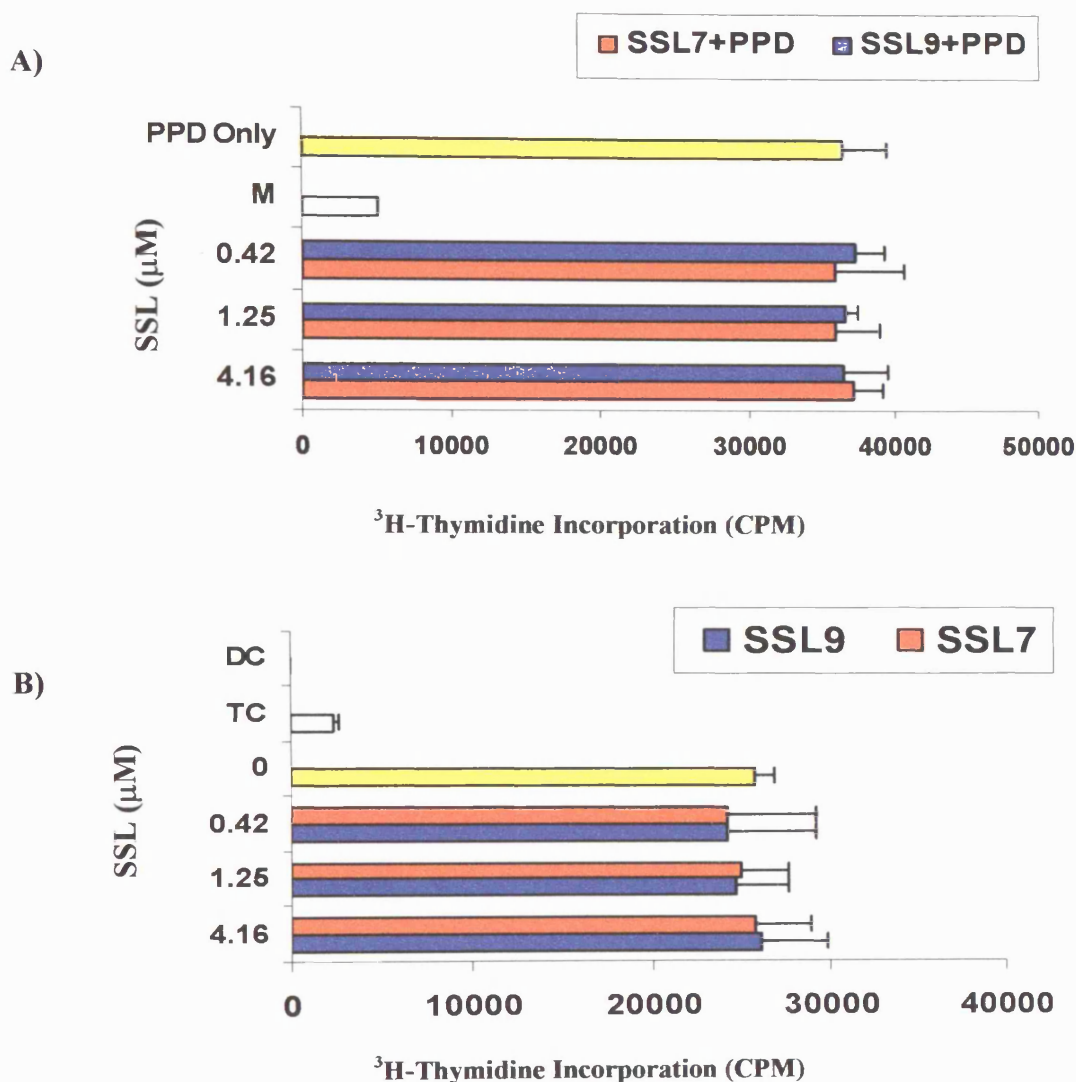


Figure 4-4. Effect of SSL proteins on T cell stimulatory capacity of DCs.

A DCs (10^4) were treated with increasing concentrations of SSL7 or SSL9 (0.42, 1.25 and 4.16 μ M), and cultured for 18 h before the addition of autologous T cells (2×10^5) in the presence of purified protein derivative (PPD; 500 U/mL). Data are mean \pm SD of 5 experiments. (M; medium).

B DCs were (10^4) treated with increasing concentrations of SSL7 or SSL9 (0.42, 1.25 and 4.16 μ M) and cultured for 18 h before the addition of allogeneic T cells (2×10^5). Data are mean \pm SD of 3 experiments. Proliferation was assessed by incorporation of 3 H-thymidine.

4.2.4 The immunological response to SSLs in the normal human population

4.2.4.1 T cell responses

To investigate the ability of SSLs to stimulate the recall T cell response in healthy volunteers, DCs were incubated with autologous T cells from normal donors in the presence of 0.42 μ M SSL7 or SSL9. Purified protein derivative (PPD) and Tetanus toxoid (TT) were used as positive controls. Figure 4-5 indicates that no or very low T cell proliferation in 6 normal volunteers was induced by these proteins. This suggests that this family of proteins has no superantigenic activity. The effect of a higher protein concentration (2.1 μ M of SSL7) on T cell proliferation was also tested. A positive T cell response was found to this concentration of SSL7 in 2 of 3 individuals (Fig 4-6).

These results highlight the possible requirement for higher *in vitro* SSL protein concentration to be processed and presented and induce a cellular T cell response. Moreover, the T cell specific for the SSL protein may be present at low precursor frequency. In the next series of experiments, therefore, the responses in eight healthy donors were studied with relatively large numbers of T cells/well (2×10^5) against DCs preloaded with higher concentrations of SSL proteins

The results of these experiments (Fig. 4-7 and 4-8) documented the presence of a recall response against this family of proteins in 3 normal subjects (37%) from the sample population (8 volunteers; 2 females, six males, age range 20-50, median approximately 30) screened. All volunteers showed a good recall response against PPD (70889 ± 3146 rpm).

Additional evidence for an antigen-specific response was also examined. T cell cytokine secretion was determined to document the type of the response specifically; whether the response is skewed towards Th1 or Th2. The supernatants of the 3 DC/T cell/SSL co-culture (individuals 1, 2 and 8 from Figs 4-7 and 4-8) were tested for IFN- γ (Th1), IL-13 (Th2), and IL-10 (Treg). All cytokine levels were low (IFN γ < 700pg/ml n=3); IL-13 < 50 pg/ml, n=3) or undetectable (IL-10). The results for the individual with maximum response (individual 1 in Fig 4-7) at different SSL concentrations are shown in Fig 4-9.

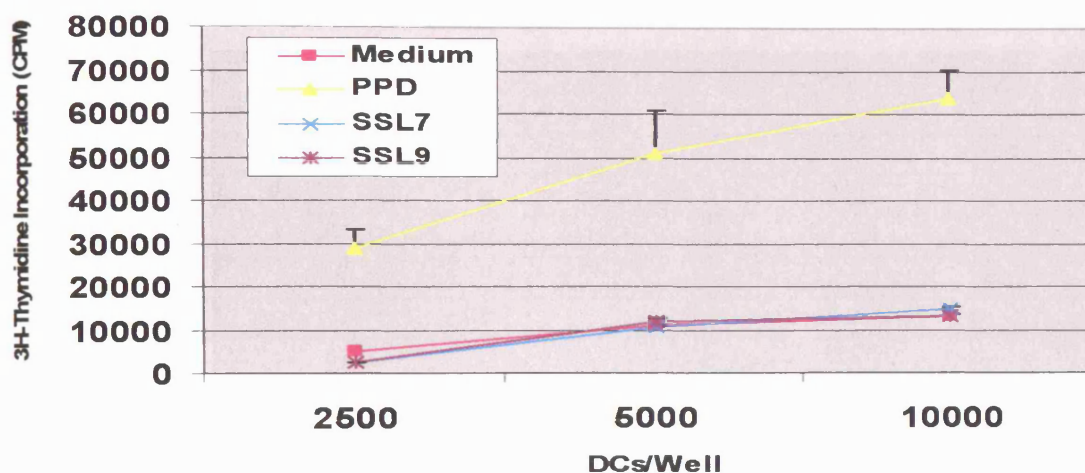


Figure 4-5. Autologous T cell responses to SSL proteins.

DCs were incubated with autologous T cells (10^6) with or without $0.42 \mu\text{M}$ of SSL proteins, and the proliferation was measured by ^3H -thymidine incorporation. Purified protein derivative (PPD; 500 U/ml) was used as positive control. Data are representative of 6 experiments carried out on cells from normal individuals

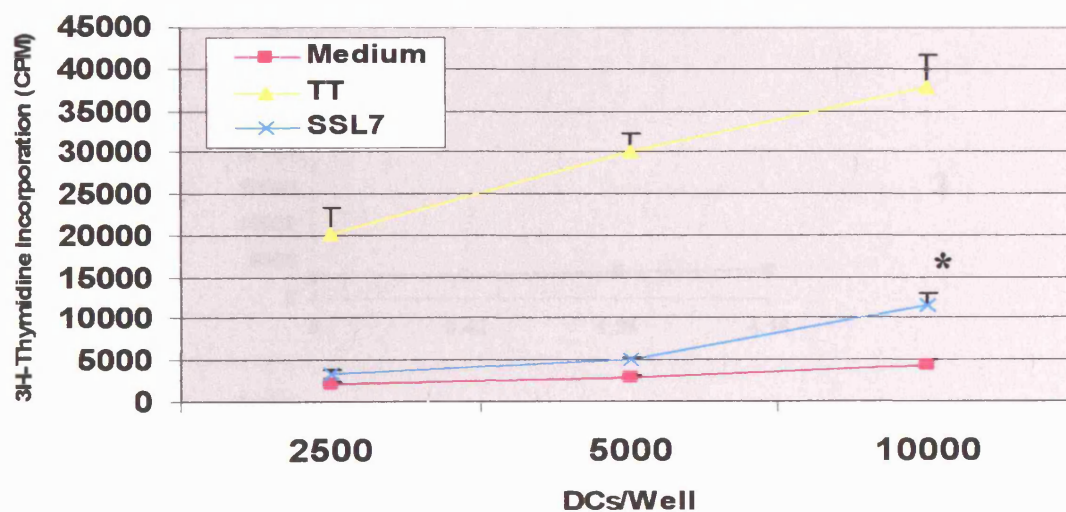


Figure 4-6. Autologous T cell responses to SSL proteins.

DCs were incubated with autologous T cells (10^6) with or without $2.1 \mu\text{M}$ of SSL7 protein, and the proliferation was measured by ^3H -thymidine incorporation. Tetanus toxoid (TT; $10 \mu\text{g/ml}$) was used as positive control. Results shown are from one experiment.

* $P < 0.05$.

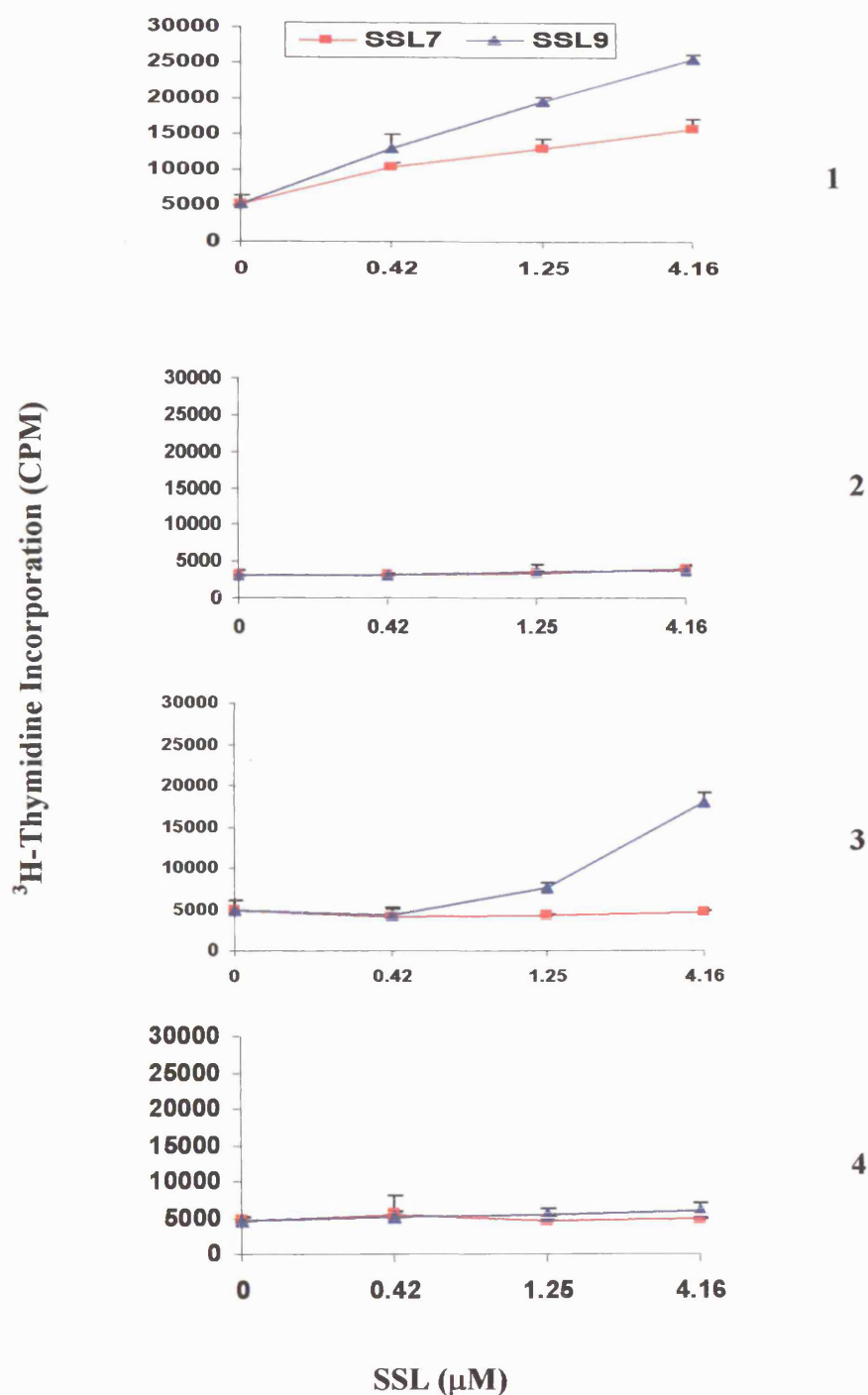


Figure 4-7. T cell responses to DC loaded with SSL protein.

DCs (10^4) were incubated for 6 days with autologous T cells (2×10^5) in presence of different concentrations of SSL7 or SSL9 (0.42, 1.25 and 4.16 μM). Data are mean \pm SD of triplicate cultures from individual experiments. Proliferation was assessed by incorporation of ^3H -thymidine.

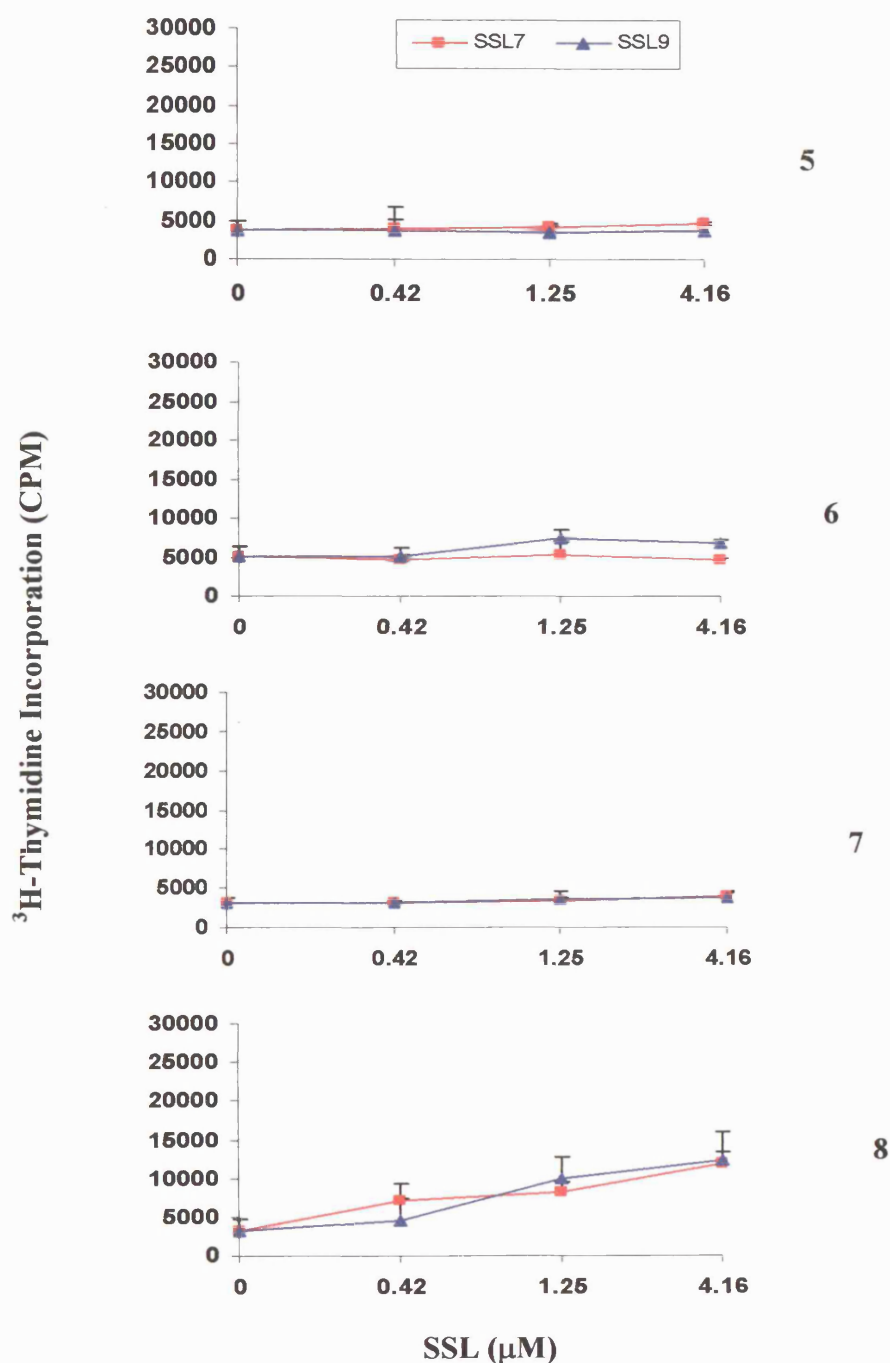


Figure 4-8. T cell responses to DC loaded with SSL protein.

DCs (10^4) were incubated for 6 days with autologous T cells (2×10^5) in presence of different concentrations of SSL7 or SSL9 (0.42, 1.25 and 4.16 μM). Data are mean \pm SD of triplicate cultures from individual experiments. Proliferation was assessed by incorporation of ^3H -thymidine.

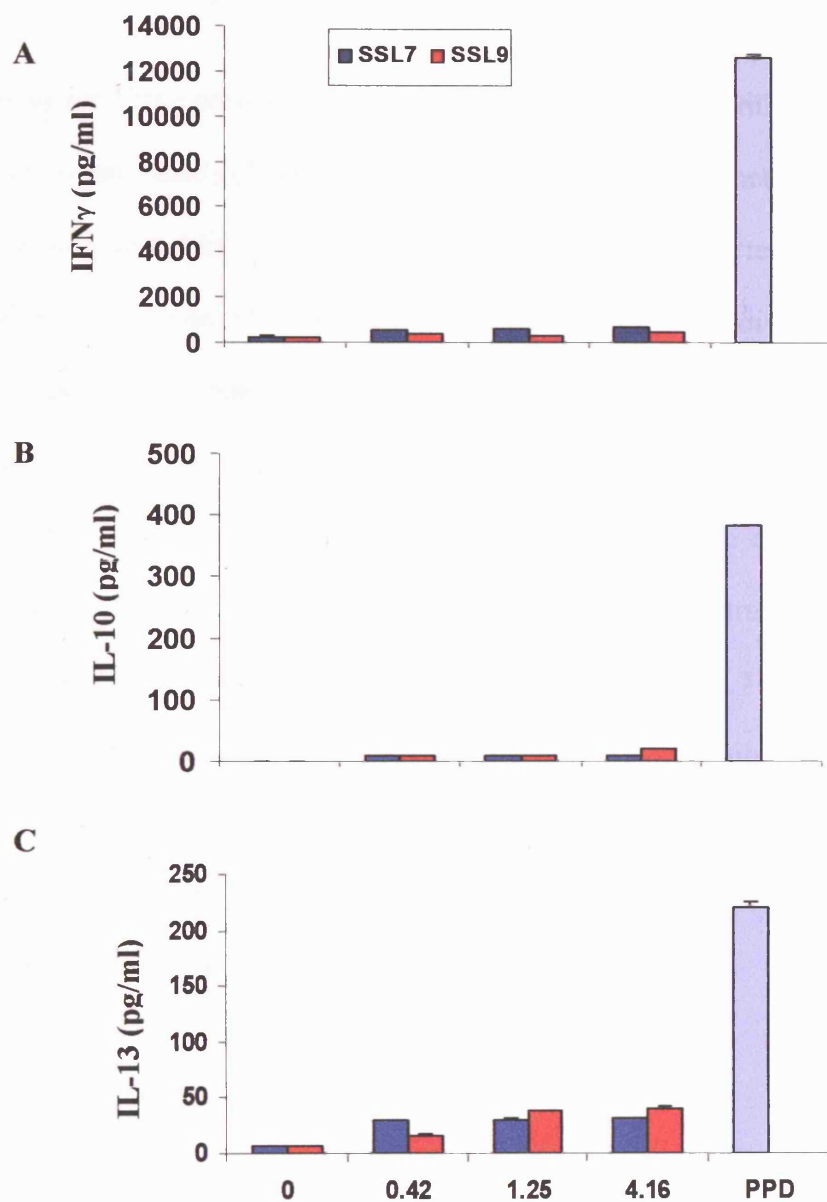


Figure 4-9. Effects of SSL protein on cytokine production.

DCs (10^4) were incubated for 4 days with autologous T cells (2×10^5) in presence of different concentrations of SSL7 or SSL9 (0.42, 1.25 and 4.16 μ M) and purified protein derivative was used as control (PPD; 500 U/mL).

4.2.4.2 *Antibodies responses*

In order to see if the presence of a T cell response correlated with antibody production, sera from 10 individuals (3 females, 7 males, age range 20-50, median approximately 30, including those tested for T cell responses as shown above) were tested by ELISA against immobilised SSL7 and SSL9 (Fig. 4-10). Nine out of ten individuals tested showed antibody responses to both SSL7 and SSL9 at this dilution, consistent with a previous report of antibodies against SSL proteins in normal volunteers (Arcus et al., 2002). Interestingly, competitive ELISA (Fig. 4-11) showed that the antibody response was highly specific for individual SSL isotypes. Increasing concentrations of SSL7 (Fig. 4-11A) were able to completely block the interaction between SSL7 protein and SSL7 sera. In contrast, neither SSL9, nor an unrelated bacterial protein (Embp32) had any effect on the SSL7 antibody binding. Conversely, only SSL9, but not SSL7 or Embp32, were able to block SSL9-antibody (Fig. 4-11B). Therefore, interaction between SSL proteins and the SSL-antibodies were specific and do not cross-react.

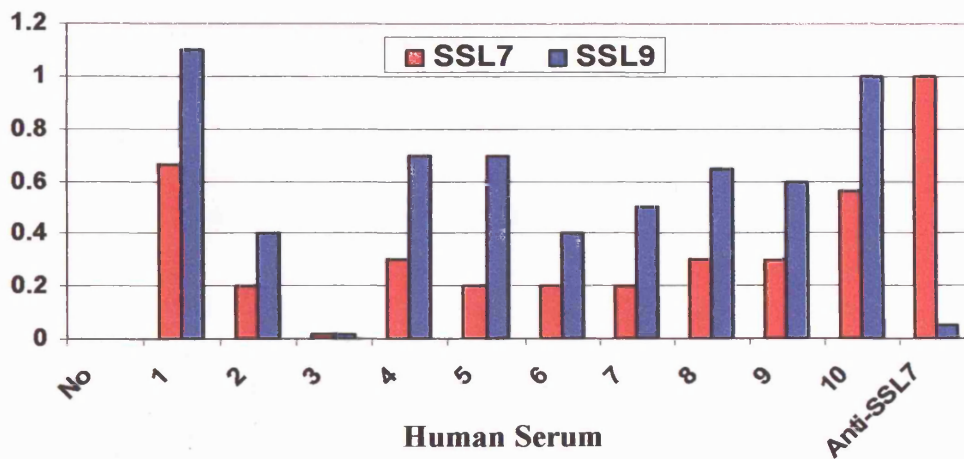


Figure 4-10. Antibody responses to SSLs in human sera.

Serum from 10 individuals was diluted by 1:2000 and tested for binding to SSL7 or SSL9 by ELISA as described in methods. Binding of an alkaline-phosphatase coupled anti human IgG was developed by adding chromogen OPD (o-Phenylenediamine dihydrochloride) and measuring the optical density at 405 nanometers. Data are representative of 3 experiments.

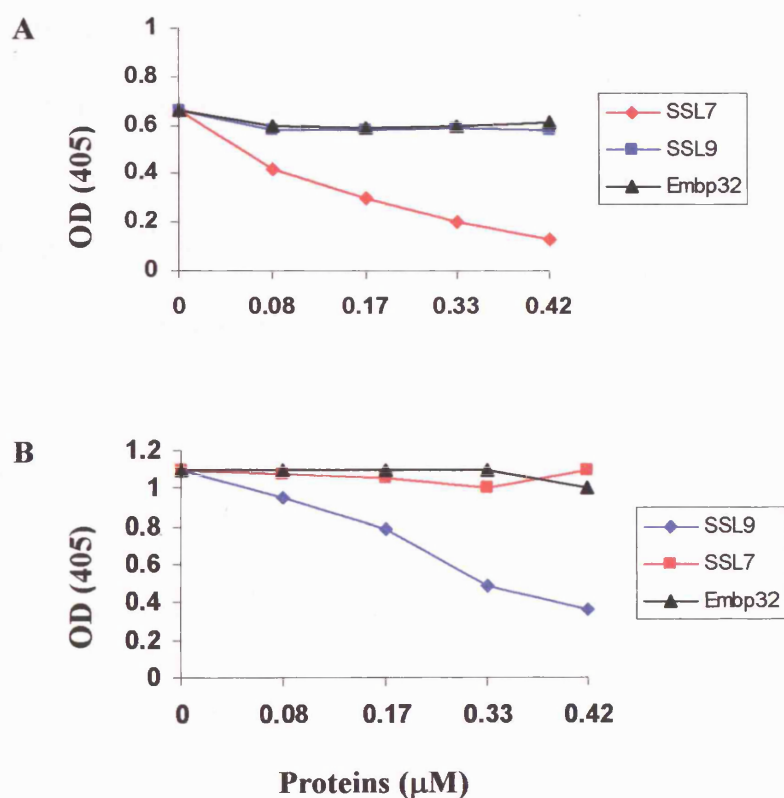


Figure 4-11. Antibody responses to SSLs in human sera.

Serum from sample 1 above (Figure 4-10) was diluted 1:2000 (final dilution) and mixed with varying concentrations of SSL7, SSL9 or Embp32 as shown. The sera were then added to plates coated with either SSL7 (A) or SSL9 (B) as shown. One experiment of two.

4.3 Discussion

We originally showed that SSL proteins have the ability to interact with cells of the immune system including monocytes and DC (see section 3.2). DCs as professional antigen presenting cells have a key role in the initiation of the immune response against microbial infections; therefore, many microbial strategies have been described which interfere with DC function (Moll, 2003). One hypothesis of SSL function suggested that these proteins may interfere with the normal function of DC and therefore impair the protective immune response to *S. aureus*. Such functions have recently been proposed for the anthrax lethal toxin (Agrawal et al., 2003) and *E. coli* heat labile toxin (Petrovska et al., 2003). This hypothesis was therefore addressed in the first part of this chapter.

SSL7 or SSL9 proteins were not toxic to antigen presenting cells nor did they alter the characteristic morphology of these cells. In contrast, dramatic alteration to DC morphology were demonstrated by *Clostridium difficile* toxin B, (Swetman et al., 2002). Conversely, SSL7 and SSL9 did not induce process extension, or up-regulation of cell surface co-stimulatory and HLA molecules on the DC, two characteristic signs of activation/maturation responses induced by whole *S. aureus* (Tourkova et al., 2001) or bacterial surface components such as PG (Michelsen et al., 2001). Thus, although SSLs bind to and are taken up by DC (see Figure 3.10) this interaction does not appear to engage activating receptors on the DC surface (similar to lectins such as mannose receptor).

In light of the potential for LPS contamination, the SSLs were expressed in a lipid A *E. coli* mutant (Somerville, Jr. et al., 1996) which makes LPS with a very low inflammatory activity. In addition a polymixin B wash step was added to scavenge any contaminating LPS (see section 2.1.1). The absence of any DC maturation in the presence of these SSLs suggested levels of LPS were below 1 ng/ml.

In addition to their specialised dendritic morphology and cell surface phenotype, DC are characterised by extremely rapid endocytosis by both fluid phase and receptor mediated uptake (Swanson and Watts, 1995; Levine and Chain, 1993). The uptake of FITC-Dx, which is believed to be mediated via mannose receptors on the cell surface (Sallusto et al., 1995) is frequently used to measure the latter. DC did indeed show efficient internalisation of FITC-Dx (albeit slightly less well after overnight culture) but this uptake was not altered by exposure to SSL7 or SSL9.

Finally, since DCs are distinguished by being the most potent stimulators of both primary and secondary T cell responses, we tested the effects of SSL7 and SSL9 exposure on DC function directly. Although DC stimulated powerful proliferative responses to both PPD (a classical recall secondary response to BCG vaccination) and allogeneic purified T cells (predominantly a primary response) neither SSL7 nor SSL9 altered the antigen presentation activity of DCs.

An alternative possibility is that SSL proteins may subvert DC activation or maturation in response to other staphylococcal components such as PG or LTA. Such functions have been documented for some bacterial toxins (see section 1.2.3). Therefore, SSLs may inhibit up-regulation of activation markers and the production of cytokines such as interleukin 12 from DCs that interacted with these modulators. This impairment could be the SSL proteins contributions to staphylococcal infections.

Next, the immune response to SSLs was analysed in a small panel of healthy human volunteers. Although none of the individuals tested had any known history of clinical *S. aureus* infection, the organism is extremely prevalent in the environment and approximately 30-40% of individuals are colonised by *S. aureus*, usually in the nasal mucosa (Nair et al., 2000). Indeed in eight volunteers tested, three (37%) showed a dose dependent T cell response to DC loaded with SSL9, and two to SSL7. The response was only detectable with relatively large numbers of T cells/well (2×10^5) and induced the release of only very low level of either Th1 (IFN- γ) or Th2 (IL-13) cytokines, suggesting that the precursor frequency of T cells specific for SSL was likely to be low.

The response to SSL7 and SSL9 was also assessed at the humoral level. Using solid phase ELISA nine out ten sera tested showed specific antibody binding to both SSLs. The response measured was IgG (using an anti-IgG detection antibody) suggesting that class switching had occurred and further implicating the activity of SSL-specific T cells. Interestingly, some individuals (e.g. individuals 2 and 7) show antibody responses but no detectable T cells responses, perhaps because precursor T cell frequency has fallen below detectable levels in these individuals. One individual showed a T cell response to SSL9,

but no antibody response to either SSL tested, although we cannot rule out that some antibody might be detectable at lower dilutions. These results agree with an earlier report of specific SSL antibody detection in human sera (Arcus et al., 2002). Interestingly, the antibody response to each SSL was highly specific with minimal evidence of cross-SSL reactivity. This data is consistent with the high sequence diversity between SSL paralogs, despite a highly conserved protein fold motif (Al Shangiti et al., 2004; Arcus et al., 2002).

Determination of the isotype profile of the anti-SSL response by using isotype specific secondary antibodies would be useful to provide alternative evidence of Th1/Th2 skewing.

CHAPTER V

**Identification of the staphylococcal superantigen like proteins
receptor(s)**

5 Identification of the SSL proteins receptor(s)

5.1 Introduction

Identification and characterization of staphylococcal superantigen-like proteins receptor(s) are important in understanding the contribution that the SSL proteins may make to *Staphylococcus aureus* infection as well as the basis of the cellular tropism of these secreted proteins.

In an attempt to isolate the receptor(s) for SSL proteins present in several cell lines, the recombinant His-tag SSL proteins were expressed, purified and immobilized using NTA-agarose affinity chromatography and allowed to interact with cell lysates from selected cell lines. Once the putative receptors have been isolated, they can be further characterized by MALDI-TOF (matrix-assisted laser desorption/ionization time-of-flight) mass spectrometry analysis or N-terminal amino acid sequencing to determine their identity.

5.2 Results

5.2.1 Screening of SSL proteins binding to different cell lines

Different cell lines were screened for the ability to bind SSL proteins (SSL7 and SSL9). Cell lines were incubated with 1.25 μ M of SSL7-FITC or SSL9-FITC for 1 h, and cell-associated fluorescence measured by flow cytometry. As demonstrated in Fig 5-1 and Table 5-1, both proteins bound to various cell lines including a human acute myelogenous leukaemia (KG1) cell line, and different EBV-transformed human B cell lines (WMPT, WMPT 3.3 and Fc7). The binding to human monocytic leukemia (U937) cells was very weak, and there was no interaction of these proteins with the rest of the screened cells, human erythroleukemic (K562), promyelocytic leukaemia (HL-60), and human acute monocytic leukemia (THP-1) cells. The fast growing cell line WMPT 3.3, which bound to SSL proteins, was used for the identification of SSL proteins receptor(s), whereas K562 cells were used as negative control.

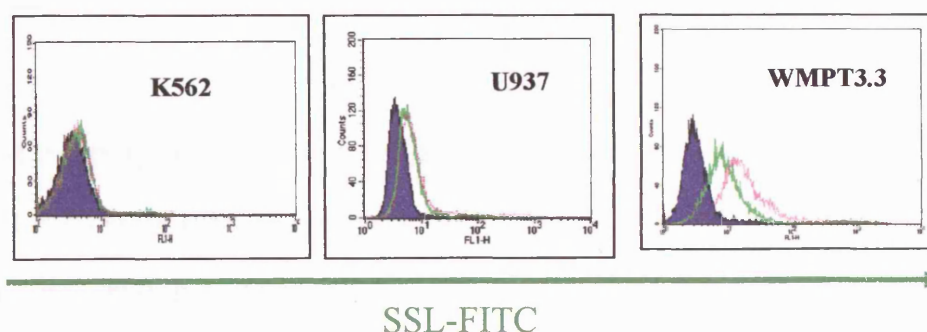


Figure 5-1. Binding of SSL7-FITC and SSL9-FITC to various cell lines

Each cell line (1×10^6 /ml) was incubated for 1 hour with 1.25 μ M of SSL7-FITC (green line) or SSL9-FITC (Pink line) at 37°C, and screened for SSL protein binding by flow cytometry. This result is representative of 3 separate experiments carried out with each cell line.

Cell lines	SSLs Binding	
	SSL7	SSL9
K562	Negative	Negative
HL-60	Negative	Negative
THP-1	Negative	Negative
U937	Positive	Positive
KG1	Positive	Positive
WMPT	Positive	Positive
WMPT 3.3	Positive	Positive
Fc7	Positive	Positive

Table 5-1. Binding of SSL7-FITC and SSL9-FITC to various cell lines

Each cell line ($1 \times 10^6/\text{ml}$) was incubated for 1 hour with $1.25 \mu\text{M}$ of SSL7-FITC or SSL9-FITC at 37°C , and screened for SSL protein binding by flow cytometry. This result is representative of 3 separate experiments carried out with each cell line.

5.2.2 Purification of SSL binding proteins using Ni-NTA agarose

In order to identify proteins which interact with SSL proteins, affinity chromatography was used to purify SSL binding proteins. Initially, recombinant SSL proteins were coupled to Ni-NTA agarose through the 6-His tag located in their amino terminus (see section 2). Two Ni-NTA agarose columns were used, one for SSL7 and one SSL9, in addition to a blank column as a control to evaluate nonspecific binding to the Ni-NTA agarose. The WMPT 3.3 cell line was expanded until it reached 1×10^9 cells, a number that was considered may be sufficient to generate enough receptor protein to identify. The cells were lysed in lysis buffer, then nuclei and cellular debris were removed by centrifugation as described earlier (2.5.1).

Proteins from cell lysates were allowed to bind to the respective columns (Ni-NTA-rSSL7 or Ni-NTA-rSSL9 or Ni-NTA) at room temperature; the columns were then washed with 10 ml of PBS. Finally, bound proteins that interact with recombinant SSLs were eluted from each column using 8M urea (pH 8), whereas SSL proteins and strongly interacting cell proteins were eluted from the Ni-NTA columns by reducing the pH of the 8M urea to 4.5. Solubilized cell proteins isolated on the Ni-NTA blank column, the SSL7-Ni-NTA column and SSL9-Ni-NTA column were collected as 1ml fractions, concentrated and separated on 10% SDS-PAGE. SDS-PAGE revealed (Fig 5-2, 5-3 and 5-4) that many proteins interacted with these proteins as well as with the blank columns. As shown in Fig 5-2 many bands with molecular weight ranging from 30-110 kDa were bound to SSL9-Ni-NTA column. These bands were enriched after concentration (Fig 5-3); two bands at around 70-80 kDa appeared to bind to SSL9-Ni-NTA (see arrow on Fig 5-3), but not to the control (see further below). No bands were visible in the elution from SSL7-Ni-NTA

by 8M urea (pH8) (Fig 5-4). A large number of proteins were eluted from both SSL7-Ni-NTA and Ni-NTA columns by 8 M urea (pH4.5).

More proteins bound to SSL9 than SSL7, this may be because the SSL9 protein has more positively charged residues (39, pI: 9.31) than SSL7 (29, pI: 6.8). Collectively these results suggested there was much non specific binding of cell lysate proteins to the Ni-NTA agarose column, making identification of a specific receptor difficult.

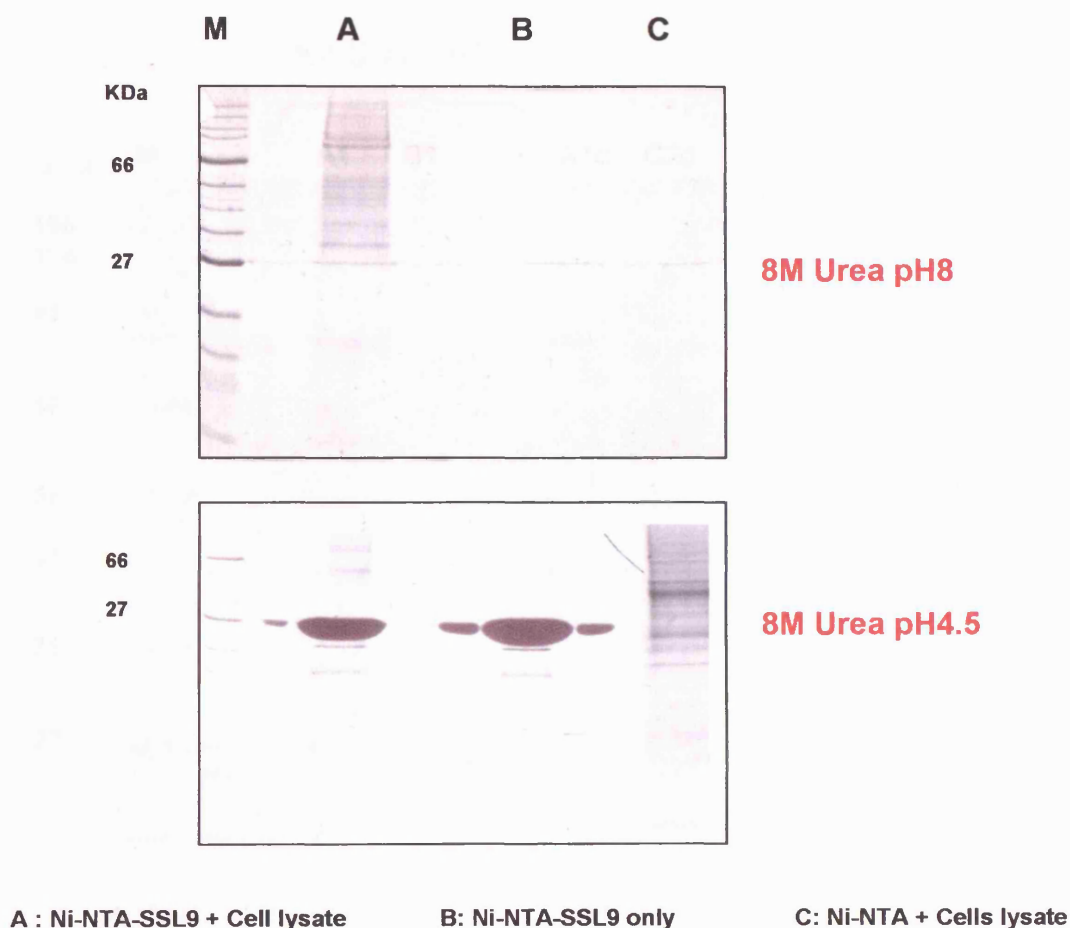


Figure 5-2. Purification of SSL9 receptors on Ni-NTA agarose columns.

SSL9 protein was coupled to a Ni-NTA agarose column as described in the Methods. WMPT 3.3 cell lysates were applied to the columns and proteins which bound to SSL9 were eluted with 8M urea (pH 8). The columns were incubated with 8M urea (pH 4.5) to elute SSL9 protein from the columns. The figures are a photograph of Colloidal Brilliant blue stained 10% SDS-PAGE.

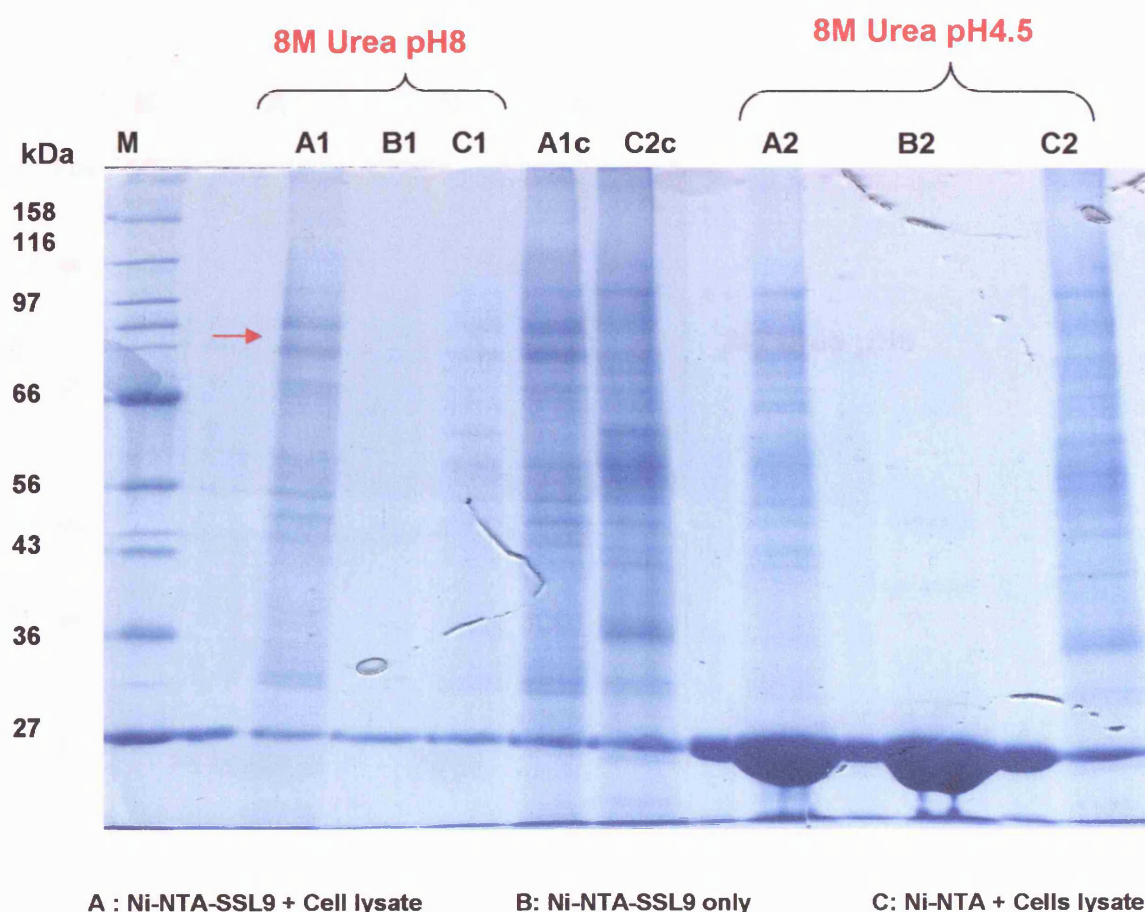


Figure 5-3. Large SDS-PAGE using 10% gel (Colloidal Brilliant blue stained).

SSL9 protein was coupled to Ni-NTA agarose columns as described in the Methods. WMPT 3.3 cell lysates were applied to the columns and the proteins which bound to SSL9 were eluted with 8M urea (pH 8). The columns were washed with 8M urea (pH4.5) to elute the SSL9 protein from the columns. Two fractions were concentrated (c) by centrifugation. The lane and numbers on the left represent molecular mass standards in kilodaltons.

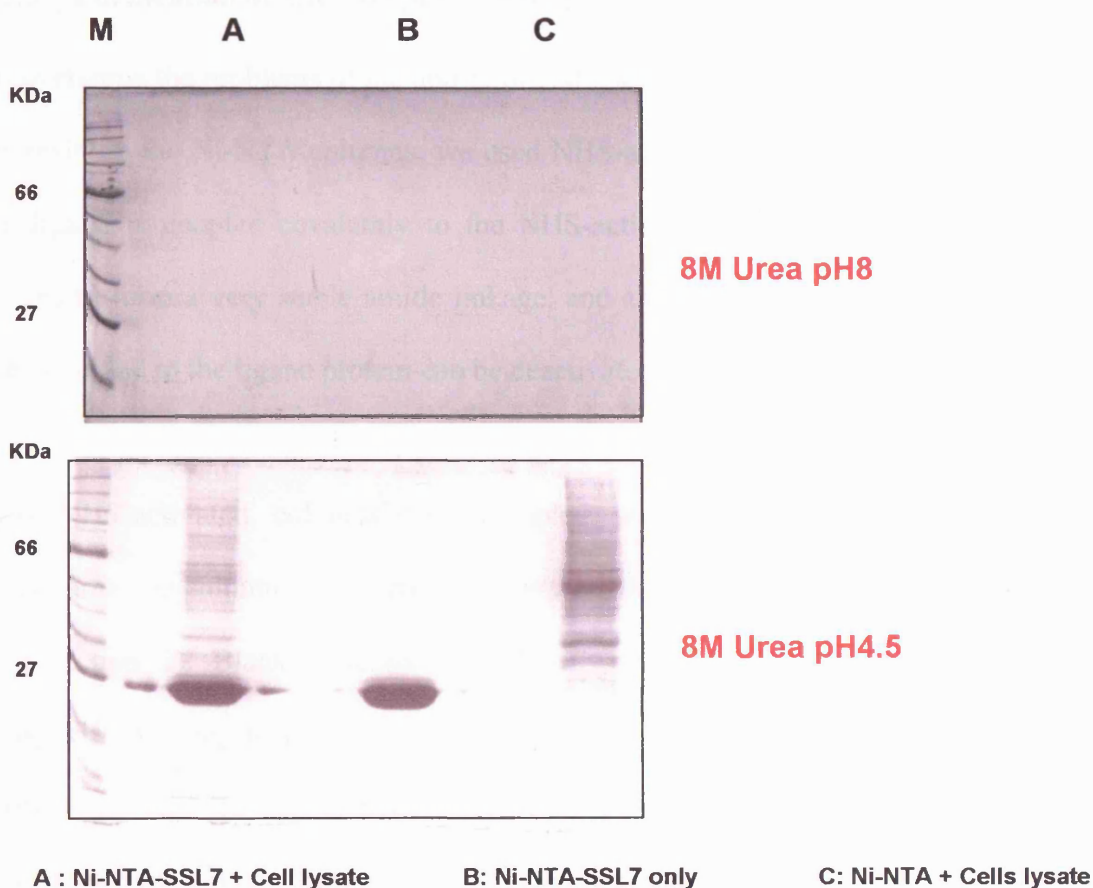


Figure 5-4. Purification of SSL7 receptors on Ni-NTA agarose columns.

SSL7 protein was coupled to Ni-NTA agarose columns as described in the Methods. WMPT 3.3 cell lysate was applied to the columns and the proteins which bound to SSL7 were eluted with 8M urea (pH 8). The columns were washed with 8M urea (pH4.5) to elute SSL7 protein from the columns. The figures are a photograph of Colloidal Brilliant blue stained 10% SDS-PAGE. The lane and numbers on the left represent molecular mass standards in kilodaltons

5.2.3 Purification of SSL receptor(s) using NHS-activated Sepharose columns

To overcome the problems of the ability of cell lysate proteins to bind to the free atoms of the resin on the Ni-NTA columns, we used NHS-activated Sepharose columns, in which the ligand is coupled covalently to the NHS-activated column through primary amino groups to form a very stable amide linkage, and any excess active groups that have not been coupled to the ligand protein can be deactivated or blocked.

Two NHS-activated columns were coupled with SSL protein (SSL7 or SSL9). Afterwards, the column's free-active groups were deactivated as described in the Methods (See section 2). Blank columns were deactivated and used as controls to evaluate nonspecific binding to the column. Total cell lysate from the WMPT 3.3 cell line was allowed to bind to the respective columns (NHS-rSSL7 or NHS-rSSL9) at room temperature for 1 hour; the columns were then washed with 10 volumes of PBS. Finally, bound proteins that interact with recombinant SSLs were eluted from each column using 0.1M glycine (pH 2.1). Solubilized cell proteins purified on SSL9 coupled affinity-column were collected as fractions (Fig. 5-5), concentrated (Fig. 5-6) and separated on 10% SDS-PAGE. Using this methodology it was possible to reduce the binding of cell lysate proteins to the column. As seen in Fig 5-6 few proteins were able to bind to the control column (NHS + cell lysate). However, many proteins were still interacting with the SSL9 protein affinity column. As mentioned above, this interaction may be a result of ionic binding between these cell lysate proteins and highly charged SSL9 protein.

In order to selectively remove those proteins binding via non-specific ionic interaction, the affinity columns were washed in a stepwise manner with 3 ml of PBS containing 0, 0.5, 1 and 2 M additional KCl, followed by elution with 0.1M glycine (pH 2.1). All fractions were collected and separated on 10% SDS-PAGE.

As demonstrated in Figure 5-7, the KCl (0.5 M) elution of the columns removed many proteins, which may represent non-specific binding. Additional faint bands, representing more tightly bound proteins, were eluted by 0.1M glycine. SSL9 binding proteins that eluted in 0.5 M KCl and 0.1M glycine were concentrated (Fig. 5-8) and separated on a large gel (10% SDS-PAGE). Despite the salt wash, a number of different bands were eluted by the acid wash, including two prominent bands around 70 kDa as seen previously in Fig 5-3. In contrast no bands were observed after elution of the SSL7-affinity columns, even after concentration of the elution fractions (Figures 5-9 and 5-10). This result is the same as that obtained using the Ni-NTA-SSL7 column (see Fig 5-4) and may be due to the weak binding between this protein and the cell line.

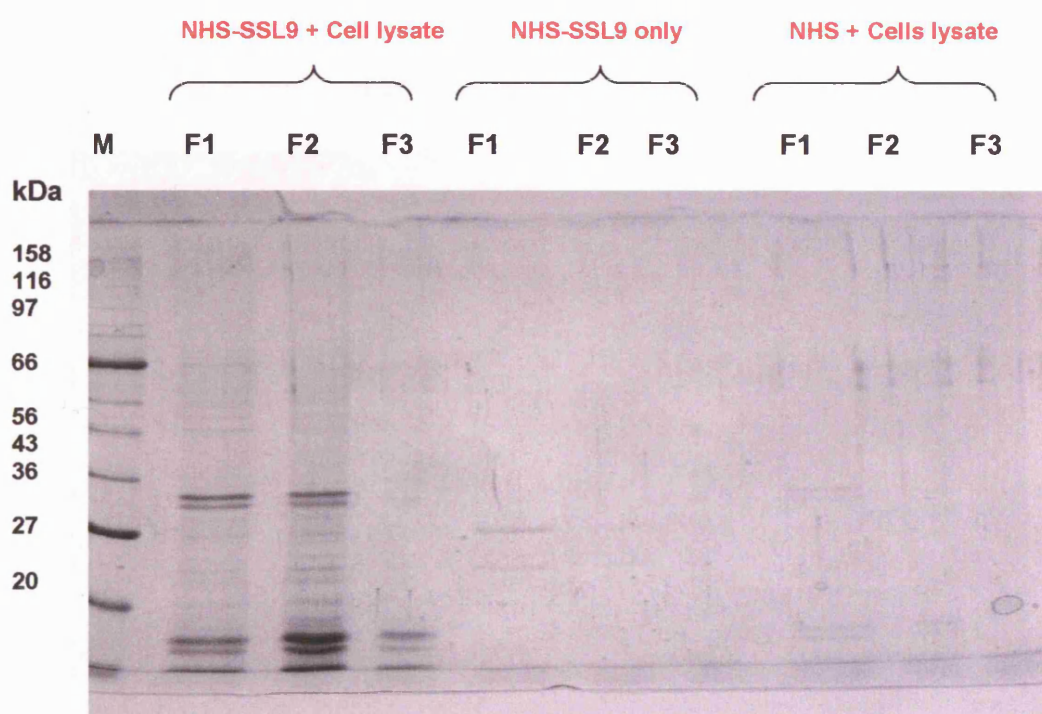


Figure 5-5. Purification of SSL9 receptors on NHS-activated Sepharose columns.

SSL9 protein was coupled to NHS-activated Sepharose columns as described in Methods. WMPT 3.3 cell lysate was applied to the columns and proteins which bound to SSL9 were eluted with 0.1M glycine (pH 2.1). The figures are a photograph of Colloidal Brilliant blue stained 10% SDS-PAGE. The lane and numbers on the left represent molecular mass standards in kilodaltons.

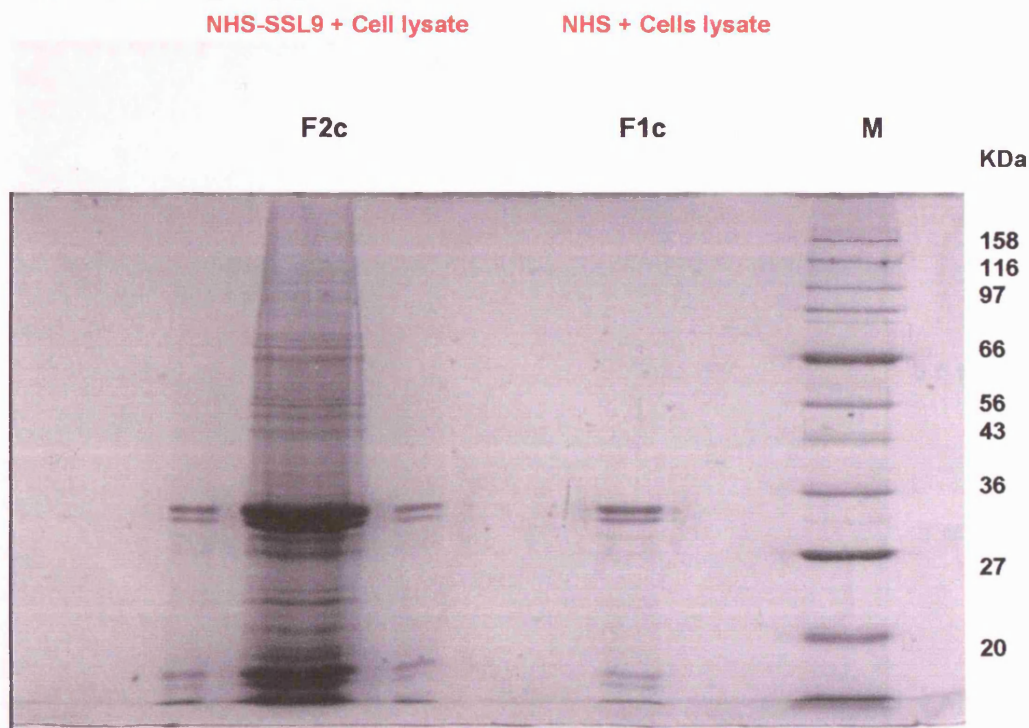


Figure 5-6. Purification of SSL9 receptors by NHS-activated Sepharose columns.

SSL9 protein was coupled to NHS-activated Sepharose columns as described in Methods. WMPT 3.3 cell lysate was applied to the columns and proteins which bound to SSL9 were eluted with 0.1M glycine (pH 2.1). The figures are a photograph of Colloidal Brilliant blue stained 10% SDS-PAGE. Fractions were concentrated (c) by centrifugation. The lane and numbers on the right represent molecular mass standards in kilodaltons.

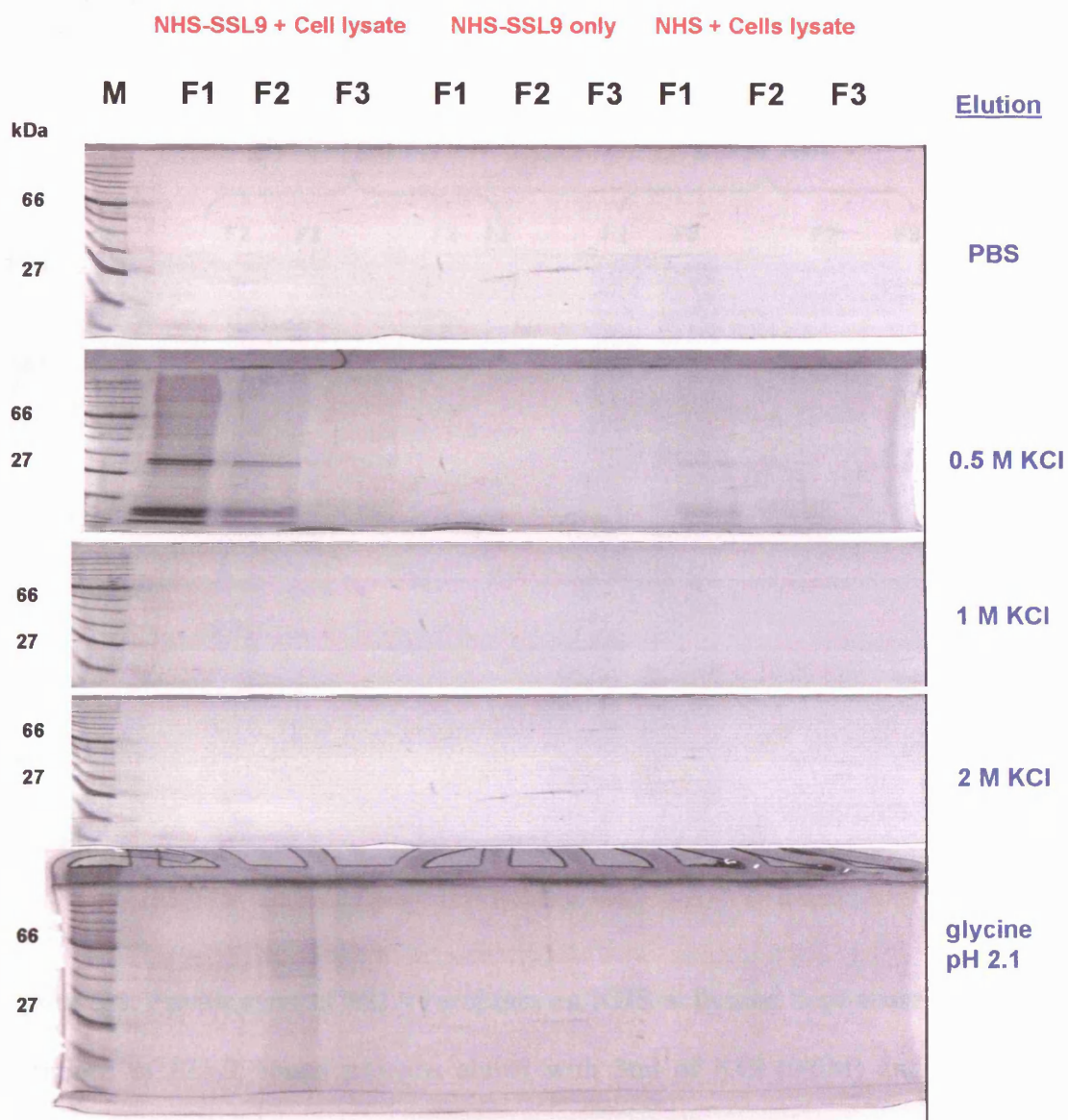


Figure 5-7. Purification of SSL9 receptors on NHS-activated Sepharose columns.

SSL9 protein was coupled to NHS-activated columns as described in Methods. WMPT 3.3 cell lysate was applied to the columns then washed with PBS. The SSL9 bound proteins were eluted with increasing concentrations of KCl (0.5, 1, 2M) followed by 0.1M glycine (pH 2.1). The figures are a photograph of Colloidal Brilliant blue stained 10% SDS-PAGE. The lane and numbers on the left represent molecular mass standards in kilodaltons

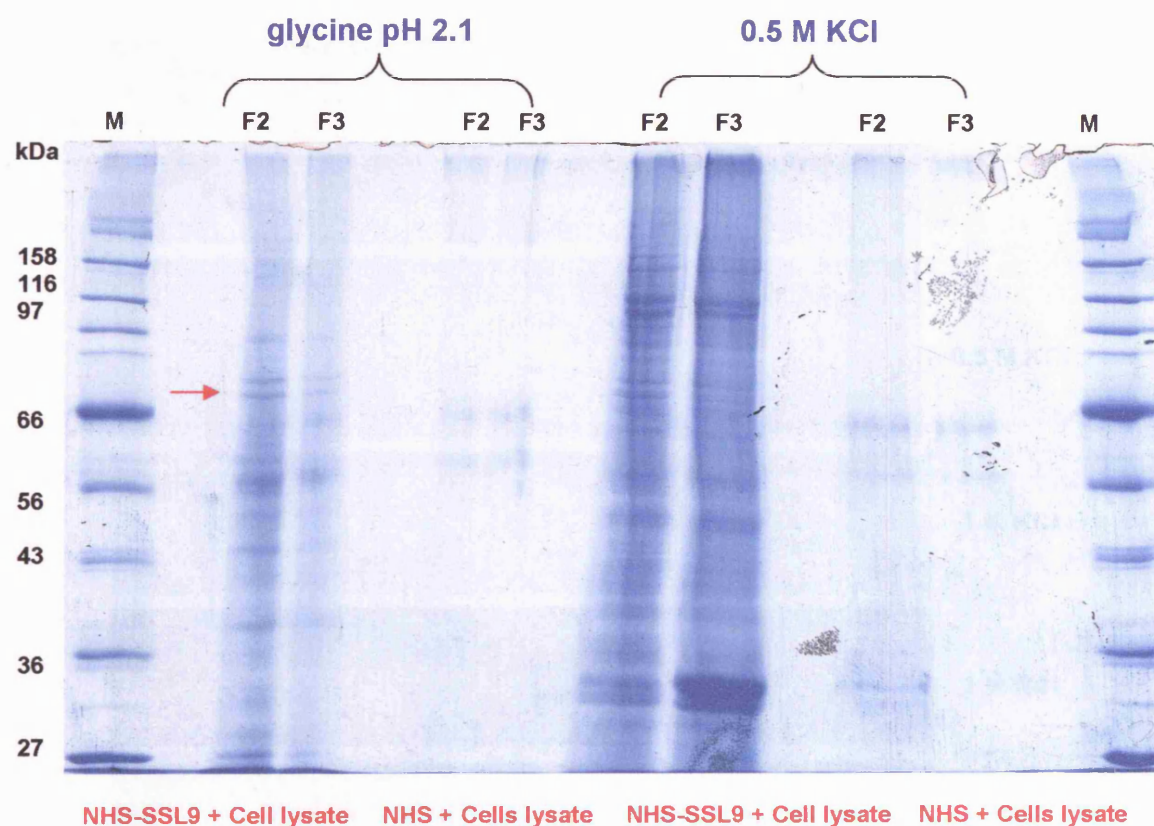


Figure 5-8. Purification of SSL9 receptors on NHS-activated Sepharose columns.

Fractions of SSL9 bound proteins eluted with 3ml of KCl (0.5M) and 3ml of 0.1M glycine (pH 2.1) were concentrated by centrifugation. The figures are a photograph of Colloidal Brilliant blue stained large 1D SDS-PAGE (10%). The lane and numbers on the left represent molecular mass standards in kilodaltons

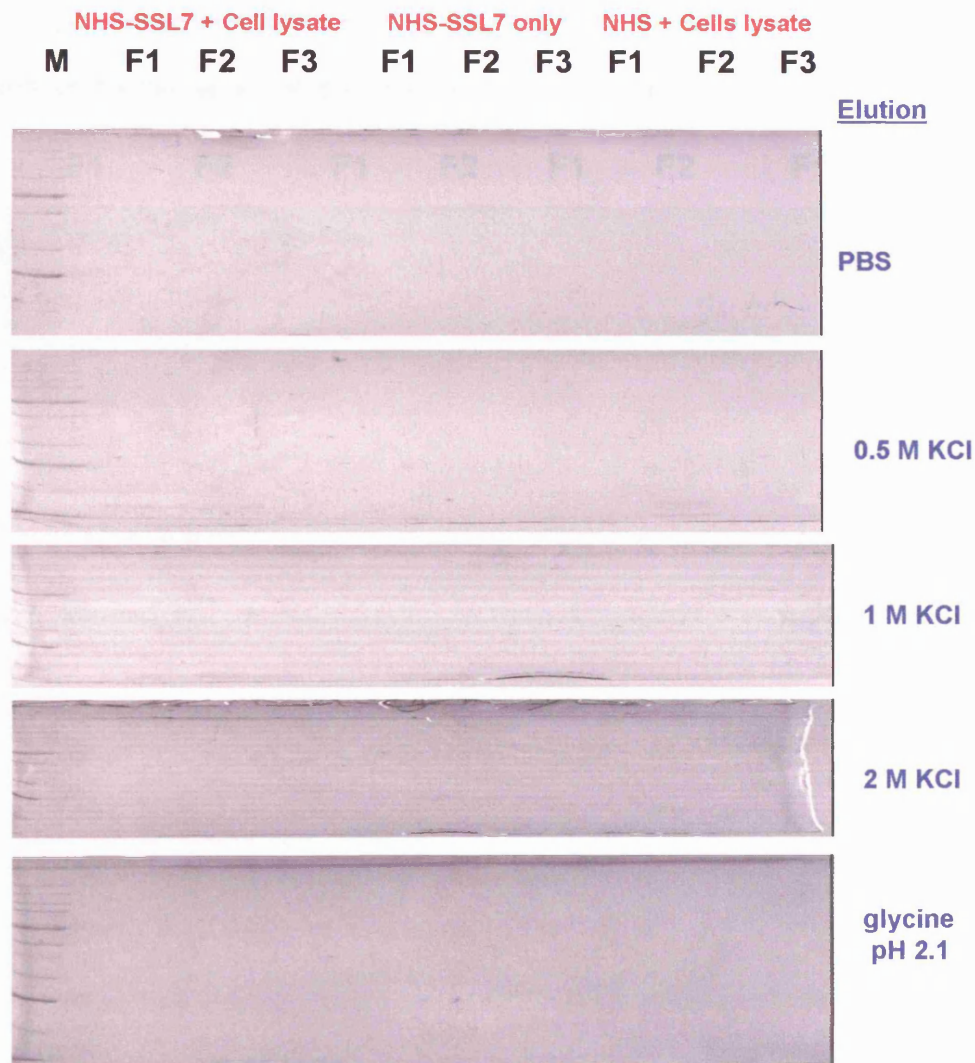


Figure 5-9. Purification of SSL7 receptors on NHS-activated Sepharose columns.

SSL7 protein was coupled to NHS-activated Sepharose columns as described in Methods. WMPT 3.3 cell lysate was applied to the columns then washed with PBS. The SSL7 bound proteins were eluted with increasing concentrations of KCl (0.5, 1, 2M) followed by 0.1M glycine (pH 2.1). The figures are a photograph of Colloidal Brilliant blue stained 10% SDS-PAGE.

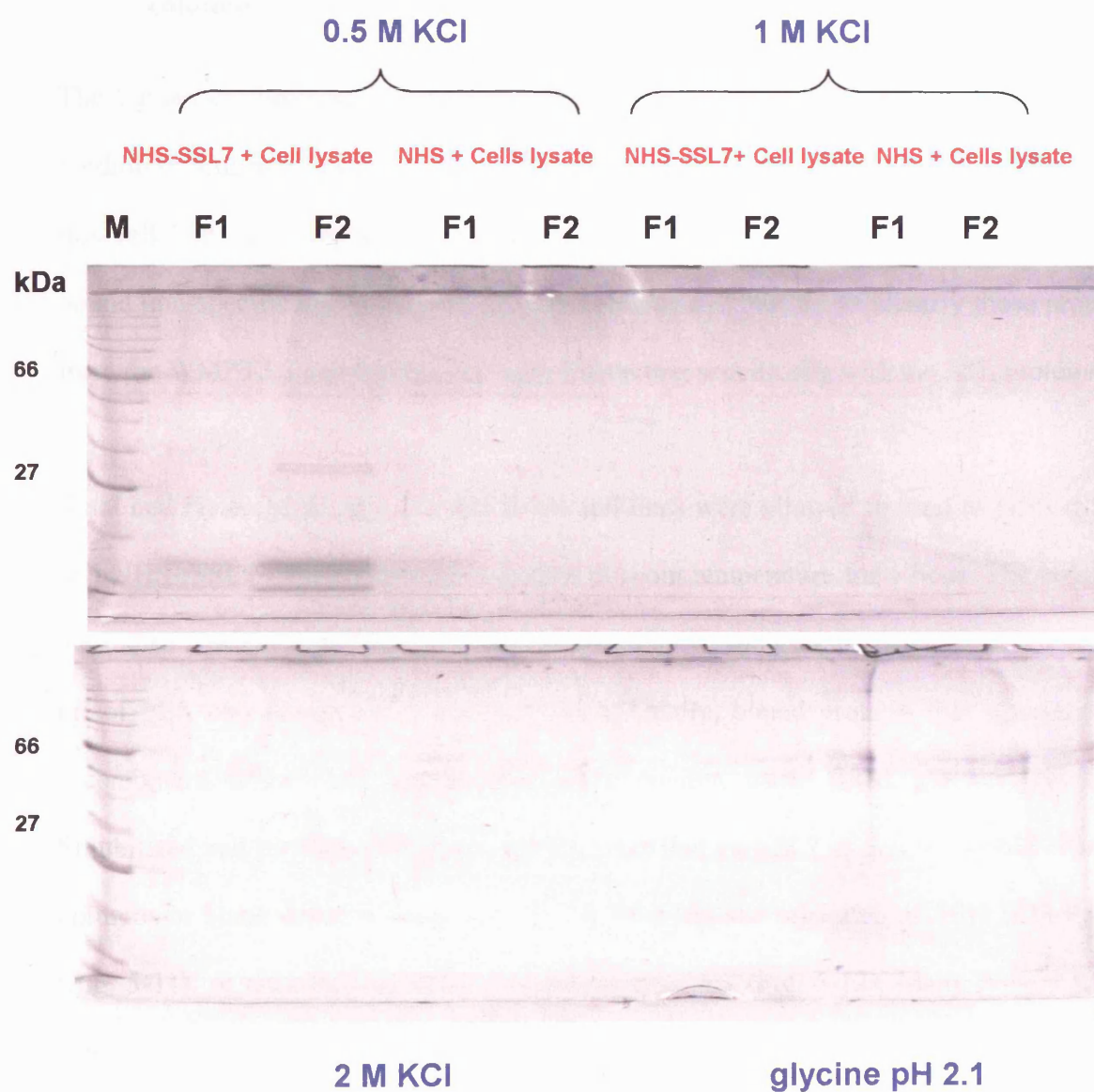


Figure 5-10. Purification of SSL7 receptors on NHS-activated Sepharose columns.

Fractions (3ml) of SSL7 bound proteins eluted with KCl (0.5, 1, 2M) and 0.1M glycine (pH 2.1) were concentrated by centrifugation. The figures are a photograph of Colloidal Brilliant blue stained 10% SDS-PAGE. The lane and numbers on the left represent molecular mass standards in kilodaltons

5.2.4 Identification of proteins which bound non-specifically to SSLs-affinity columns

The human erythroleukemic (K562) cell line was among the cell lines that showed no binding to SSL proteins as demonstrated by flow cytometry (see section 5.1.2). Therefore, this cell line was used as a negative control to identify proteins in cell lysates which bound non-specifically to the SSL affinity columns and thus try to identify those proteins from the WMPT3.3 cell lysates that were interacting specifically with the SSL proteins.

Total cell lysate of WMPT 3.3 and K562 cell lines were allowed to bind to NHS-rSSL7 and NHS-rSSL9 column or blank columns at room temperature for 1 hour. The columns were then washed with 10 ml of PBS, followed by washing in a stepwise manner with 3 ml of PBS containing 0.5, 1 and 2 M KCl. Finally, bound proteins that interact with recombinant SSLs were eluted from each column using 0.1M glycine (pH 2.1). Solubilized cell proteins from both cell lines purified on SSL7 or SSL9 coupled affinity-columns or blank columns were collected as fractions and separated on 10% SDS-PAGE (Fig. 5-11), or concentrated and separated on large gel (Fig. 5-12). Many protein bands were observed after concentration of elution fractions from both cell line lysates. However, a single clear band was visible in WMPT3.3 eluates but not from K562 eluates, with a molecular weight of around 70 kDa (Fig. 5-12).

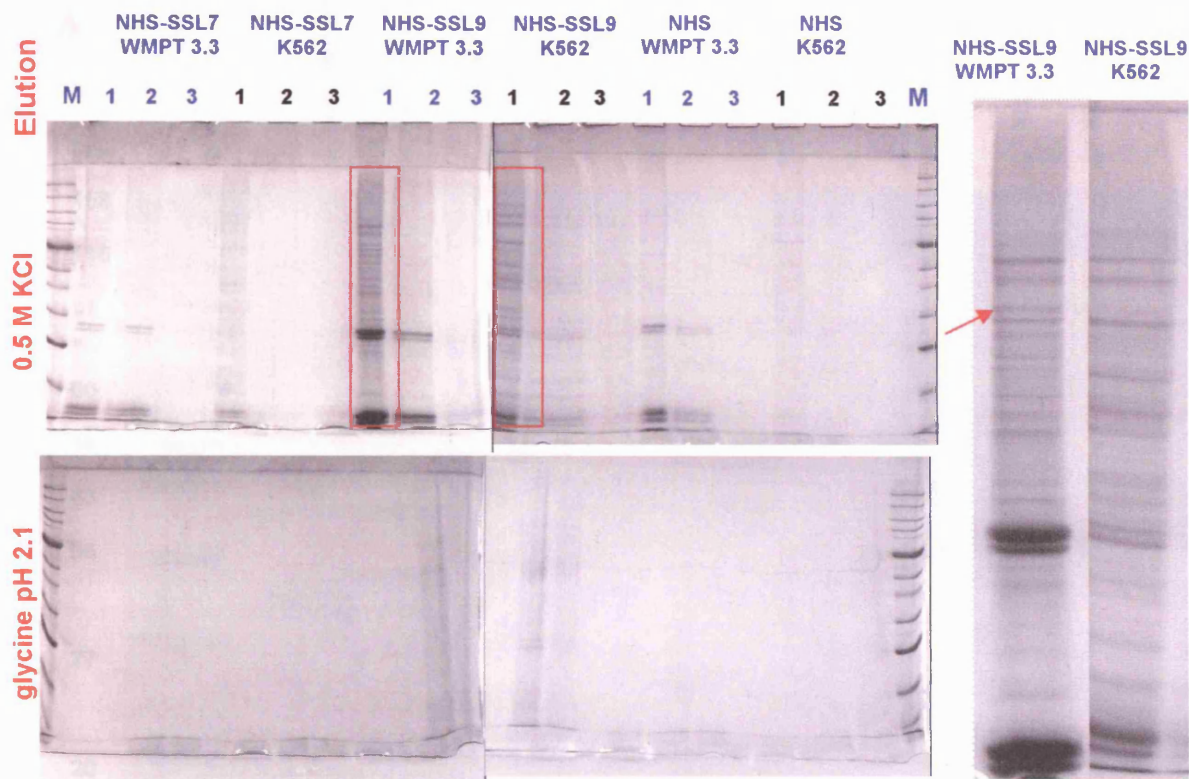


Figure 5-11. Purification of SSLs receptors on NHS-activated Sepharose columns.

SSL7 and SSL9 proteins were coupled to NHS-activated columns as described in Methods. WMPT 3.3 or K562 cell lysates were applied to the columns then washed with PBS. The SSL7 bound proteins were eluted with increasing concentrations of KCl (0.5, 1, 2M) followed by 0.1M glycine (pH 2.1). The figures are a photograph of Colloidal Brilliant blue stained 10% SDS-PAGE. Figure on the right is enlargement of selected areas. The arrow indicates the 70 kDa band of interest.

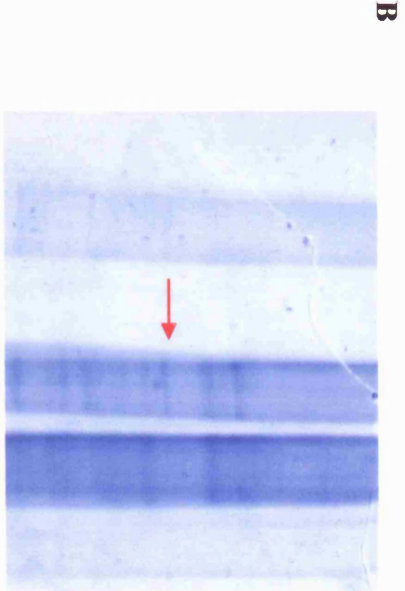
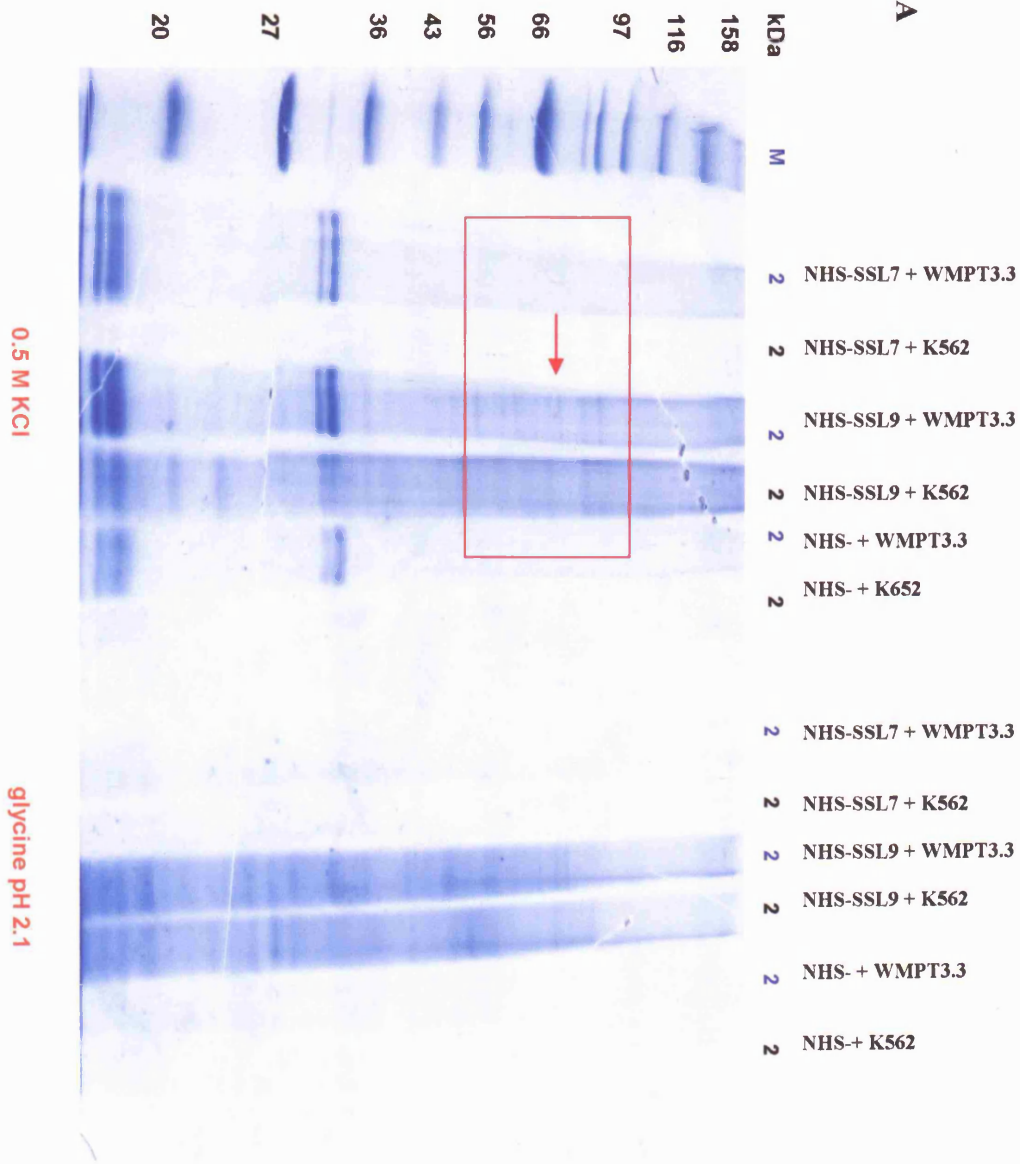


Figure 5-12. Purification of SSLs receptors by NHS-activated Sepharose columns.

A) SSL7 and SSL9 proteins were coupled to NHS-activated columns as described in Methods. WMPT 3.3 or K562 cell lysates were applied to the columns then washed with PBS. The SSL bound proteins were eluted with KCl (0.5M) and 0.1M glycine (pH 2.1) and concentrated by centrifugation. The arrow indicates the 70 KDa proteins of interest. The figures are a photograph of Colloidal Brilliant blue stained large SDS-PAGE (10%). The lane and numbers on the left represent molecular mass standards in kilodaltons.

B) Enlargement of selected area.

5.2.5 Protein identification by peptide mass fingerprinting

In order to identify the protein band obtained from large gel (Fig 5-12), the 70 kDa band was excised and subjected to tryptic digestion, and the resulting peptide mixture was analysed by MALDI-TOF mass spectrometry (carried out by Dr S. Howell, NIMR, London). Using this method we identified the protein as heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa) or BiP (Table 5-2).

Table 5-2. Protein identified by MALDI-TOF fingerprints

Accession no.	MOWSE Score	Mass	Protein Description
16507237	7.198e+04	72402	Heat shock 70kDa protein 5
	(Protein Prospector)		(glucose-regulated protein, 78kDa); BiP

5.3 Discussion

Cellular infection by bacteria is a multistep process, whereby some bacteria or bacterial products attach to the cell via host cell surface molecules and then initiate the internalization process. We suggested previously (see section 3.2.1) that the SSL proteins bind to cells of immune system through specific but different binding partners or binding sites. In an attempt to identify the putative receptors for this family of proteins from selected cell lines, we used affinity chromatography and mass spectrometry to isolate and identify the SSL receptors. Proteomic analysis revealed that heat shock 70kDa protein 5 (glucose-regulated protein 78kDa (GRP78) also known as BiP (heavy chain binding protein), was a candidate receptor for SSL9.

Cells exposed to stressful conditions, such as high temperatures, oxidative injury, heavy metals, and proinflammatory cytokines, increase synthesis of a multifunctional group of proteins referred to as stress proteins or heat shock proteins (HSPs). They have been designated as a member of the larger family of proteins called molecular chaperones (Moseley, 2000). These proteins are classified into families according to their molecular mass (ranging from 110 to 8 kDa), and serve as cellular chaperones, participating in protein synthesis and transport through the different cellular compartments, and can bind a wide variety of targets (for review see (Kiang and Tsokos, 1998; Srivastava, 2002)).

HSPs are among the most abundant intracellular cytoplasmic molecules present in both eukaryotic and prokaryotic cells, under both unstressed and stressed conditions (Moseley, 2000). However, several studies have suggested that some members of this family such as HSP60, HSP70 and HSP90 are also expressed on the surface of cells (Arap et al., 2004; Guzhova et al., 1998; Multhoff and Hightower, 1996; Triantafilou et al., 2002).

BiP (GRP78) was originally identified independently as the immunoglobulin heavy chain binding protein (Haas and Wabl, 1983) and as the glucose regulated protein (Pouyssegur et al., 1977). Like other HSP70 proteins, BiP has two major domains, an N-terminal and C-terminal domains, with 61% and 66% amino acid sequence identity with that of HSP70 and HSC70 proteins, respectively. The C-terminal binding domain fold of BiP is similar in all members of the HSP70 protein family (Gething, 1999; Munro and Pelham, 1986). *grp78* gene expression is increased in response to cellular stresses, including glucose starvation, accumulation of unglycosylated proteins and oxygen deprivation (Gething, 1999). BiP is a resident luminal endoplasmic reticulum (ER) protein that binds to patches of hydrophobic amino acids (Flynn et al., 1991; Foy and Matsuuchi, 2001). It has several function, including acting as a molecular chaperone in antigen presentation, catalysing the folding and translocation of nascent polypeptide chains including the folding and assembly of MHC I molecules, and it has also been suggested that it protects cells from ER stress (Morris et al., 1997).

Several studies have shown that BiP (GRP78) can also be expressed on the cell surface (Arap et al., 2004; Delpino and Castelli, 2002; Shin et al., 2003). Shin et al (2003) (Shin et al., 2003) confirmed an abundance of ER chaperone proteins (including BiP) on the cell surfaces of five different cell lines. Interestingly, BiP (GRP78) has recently been identified as a co-receptor protein for non-enveloped Cocksackievirus A9 (Triantafilou et al., 2002). In this system, BiP acts as a binding protein for the virus while MHC class I mediate virus internalization. More recently, Jindadamrongwech et al (2004), (Jindadamrongwech et al., 2004) have demonstrated that BiP also functions as receptor for dengue virus serotype 2.

The experiment which identified BiP as a candidate receptor for SSL proteins was undertaken only once due to the project time limits, so further experiments to confirm these finding are needed. In addition to repeating the biochemical isolation, several additional pieces of evidence are required to confirm whether BiP (GRP78) is indeed the SSL receptor. These will include examining whether BiP is expressed on the surface of monocytes and DCs by flow cytometry. Further validation should include transfection of a BiP cDNA into a non-binding cell line (such as K562 or T cell) to demonstrate the conversion of the cell line to a binding one. Antibodies against BiP may also be tested for their ability to block SSL binding.

At this stage, identification of BiP as the SSL receptor must be considered preliminary, especially as the ability to bind 'non-specifically' to many proteins is one of the characteristic features of this chaperone family.

Nevertheless, binding of bacteria with ER proteins has been documented previously. Talilleux et al (2003) (Tailleux et al., 2003) have found that a significant proportion of mycobacterial phagosomes stained strongly for the ER-specific markers BiP (GRP78) in antigen presenting cells and suggested this co-localization could be a specific mycobacterial ER-targeting strategy.

In a different context, it has been reported that monocytes become apoptotic following phagocytosis of *S. aureus*, and that this bacterium can induce selective expression of HSP70 in these cells (Kantengwa and Polla, 1993). Moreover, it has been reported that the expression of HSP72 provides efficient protection against apoptosis triggered by phagocytosis of *S. aureus* by monocytes (Guzik et al., 1999). So, it is tempting to speculate that the binding of SSL proteins to this chaperone family might be a bacterial strategy to block a protective activity provided by chaperones to the immune cells.

CHAPTER VI

General Discussion

6. General Discussion

The major role of the immune system is to identify and eliminate pathogens. Cells of the immune system are well equipped for sensing pathogens and their products or danger signals delivered from infected cells. DCs have been recognised as an important cell population that are able to link pathogen detection with regulation of the adaptive immune responses. Many microbial strategies have therefore evolved to induce alterations in the functional properties of these professional antigen presenting cells, preventing their normal function and thus moderating the response of the host to the bacterium (Moll, 2003).

The pathogenicity of *Staphylococcus aureus* is multifactorial, generally involving a large number of extracellular proteins. Some of these proteins, including cytotoxins and exoenzymes, are secreted; others, including protein A and various adhesins, remain attached to the cell wall. Together, these proteins enable the organism to evade host defences, adhere to host cells and intercellular matrix molecules, invade or destroy host cells, and spread within the tissues (Proft and Fraser, 2003).

Production of many different exotoxins by a single bacterium may reflect the nature of the multiplication strategy of *S. aureus* in the human body (Kuroda et al., 2001). Several bacterial species have developed a system of evading the host immune system as a microbial strategy for survival. However, *S. aureus* seems to challenge the immune responses of the host by initiating local inflammation and subsequent abscess formation.

This localization of bacteria inside the abscess may help *S. aureus* to concentrate the secreted proteins and enzymes which have the ability to destroy the cells of immune systems and surrounding tissues.

The number of staphylococcal SAgS identified has increased due to various *S. aureus* genome sequencing projects (Baba et al., 2002; Fitzgerald et al., 2003; Kuroda et al., 2001; Orwin et al., 2001; Orwin et al., 2003). In 2000, a novel gene cluster in the staphylococcal genome was identified that harboured at least five related genes with the characteristic SAg family signature. The corresponding gene products were originally named staphylococcal enterotoxin-like toxins (SET) (Williams et al., 2000). Members of this protein family were recently renamed the staphylococcal superantigen-like (SSL) proteins (Lina et al., 2004). Two SSLs (SSL5 and SSL7) have been crystallized and the structure determined revealing a typical SAg fold. However, so far none of the SSLs have exhibited any of the functional hallmarks of all SAgS, such as MHC class II binding or T cell stimulation. Thus, while they are structurally related, they appear to have very different functions (Al Shangiti et al., 2004; Arcus et al., 2002).

The function of SSLs is still unknown, but their location within a pathogenicity island in all *S. aureus* strains tested so far, as well as the presence of SSL specific immunity in so many individuals, and the existence of so many SSL paralogs in the *S. aureus* genome, is suggestive of a strong interaction between host immunity and evolution of this bacterial family of proteins. One possibility is that SSL7 and SSL9 induce alterations in the functional properties of these cells, preventing their normal function and thus moderating the response of the host to the bacterium. An alternative possibility, however, is that like classical superantigens, SSLs may allow *S. aureus* to stimulate a non-protective immune

response and thus to distract the protective adaptive immune response of the host, and contribute to bacterial pathogenicity. The uptake of SSL7 and SSL9 into an endosomal compartment which intersects with the dextran uptake pathway is certainly compatible with such a role, since uptake via the mannose receptor efficiently targets antigens to the Class II MHC antigen processing pathway (Sallusto et al., 1995).

Limited information is available on the direct effect of *S. aureus* and its toxins - apart from SAGs - on DCs, in particular on their subsequent ability to induce T cell proliferation, which is a fundamental step in the human adaptive immune system. This information is important because it may highlight the cellular interaction occurring during innate as well as adaptive immune responses against *S. aureus* in the course of infection. Thus, we studied the ability of SSL treated DCs to stimulate recall T cell responses in healthy individuals as well as the ability to stimulate responses to SSLs themselves. T cell recall responses to SSLs were observed in some individuals, complementing the known antibody response to these proteins.

A number of bacterial strategies have been documented by which bacteria avoid detection and elimination by the immune system via interfering with DC function (described in the general introduction, see section 1.2.3). We have found that SSL proteins (SSL7 and SSL9) fail to up-regulate DC's surface activation markers or their endocytic activity. Furthermore, DCs treated with SSL proteins have the same antigen presentation activities to stimulate both allogeneic response (processing independent responses) and a recall response to PPD (processing dependent). Therefore, the antigen presenting cell activity is not adversely affected by the presence of SSLs. Conversely, self-targeting to antigen presenting cells may result in enhancing the immunogenicity of these proteins. Although

enhancing immunogenicity would, at first sight, appear to be paradoxical, the generation of an antibody response to a secreted protein is unlikely to confer any advantage to bacterial clearance by the host. On the contrary, the interaction between secreted toxin and specific antibody in the microenvironment of the bacterium may activate complement and hence contribute to the breakdown of the physical barriers which restrict the invasiveness of these bacteria.

The final chapter of the thesis describes a preliminary identification of BiP (GRP78), a glucose-regulated protein belonging to the heat shock 70 family of proteins, as candidate receptor for SSL proteins. BiP has been identified as a viral receptor and has been implicated as chaperone in antigen presentation and delivery of peptides to MHC molecules in ER lumen. In view of our finding one could suggest a model wherein SSL utilizes BiP, a MHC-associated protein, as receptor or binding molecule for attachment to the cell surface followed by internalisation and thus gains access to the cell's machinery. The antigens enter the endolysosomal pathway, where proteins are digested and load onto class II molecules for presentation to T cells. So, it is tempting to speculate that the binding of SSL proteins to this chaperone would be a bacterial strategy to prevent antigen presentation activity of the immune cells. One example of immune evasion carried out in this manner is the bacterium *H. pylori*, which secret vacuolating protein toxin (VacA) (Reyrat et al., 1999) that causes vacuolation of acidic endocytic compartments; this elevation of endolysosomal pH decreases lysosomal protease activity. Therefore, the generation of class II-specific peptides may be impaired, as may the proper proteolytic cleavage of the invariant chain molecules (Satin et al., 1997; Molinari et al., 1998; Hudson and Ploegh, 2002).

In conclusion, this study describes some of the functional consequences of the interaction between SSL and DC. In contrast to some other bacterial exotoxins (Agrawal et al., 2003; Petrovska et al., 2003) SSLs do not appear to damage DC, but rather can be taken up by them, and thus stimulate T cell responses in a significant fraction of healthy individuals. Further studies will be necessary to demonstrate whether this immune response is of potential benefit to the bacteria or the host. Taken together, therefore, these data do not provide any evidence that SSL proteins inhibit or modify DC function, although we cannot rule out the possibility that they act on some function as yet untested.

Future work

This investigation has generated many questions that would be of interest to follow up through further experiments. Some of the possible future directions have already been addressed in relevant chapters. Here some outline strategies for future work that may be of particular interest.

- Assessment of the effect of SSL proteins on monocyte differentiation to DC as well as antigen presentation capacity of monocytes to activate allogeneic T cell proliferation and cytokine production.
- Comparison of the immunological response to SSL proteins in individuals with recent clinical *Staphylococcus aureus* infection to those from the normal population.
- Characterisation of the B cell sub-population which interacts with SSL7.
- Examination of the expression of BiP (GRP78) protein on the surface of monocytes and DCs and assessment of the effect of antibodies against this protein to block SSL/DC binding.
- Assessment of the effect of SSL proteins on DC phagocytosis to whole *S. aureus* bacteria and its migration ability.
- Investigation of the ability of these proteins to bind mouse DCs, an approach to *in vivo* studies.

CHAPTER VII

References

7. References

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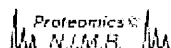
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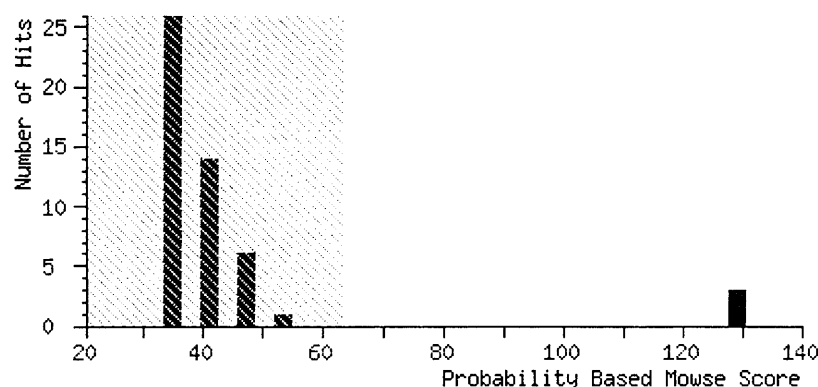


Mascot Search Results

User : Ali
Email :
Search title : 1
Database : NCBIInr 20040105 (1590839 sequences; 520945819 residues)
Taxonomy : Homo sapiens (human) (101738 sequences)
Timestamp : 7 Dec 2004 at 16:26:58 GMT
Top Score : 129 for gi|6470150, BiP protein [Homo sapiens]

Probability Based Mowse Score

Score is $-10 \cdot \log(P)$, where P is the probability that the observed match is a random event. Protein scores greater than 63 are significant ($p < 0.05$).



Protein Summary Report

To create a bookmark for this report, right click this link: [Protein Summary Report \(1\)](#)

[Re-Search All](#)
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Index

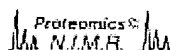
Accession	Mass	Score	Description
1. gi 6470150	71002	129	BiP protein [Homo sapiens]
2. gi 87528	72185	128	dnaK-type molecular chaperone HSPA5 precursor - human
3. gi 16507237	72402	128	heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa); BiP
4. gi 37546003	39484	52	hypothetical protein XP_353073 [Homo sapiens]
5. gi 563444	11055	50	Ig heavy chain (VH4) V region (VDJ) [Homo sapiens]

Results List

1. gi|6470150 Mass: 71002 Score: 129

BiP protein [Homo sapiens]

Observed	Mr(expt)	Mr(calc)	± ppm	Start	End	Miss	Peptide
997.535	996.527	996.510	17.2	280 -	288	0	ALSSQHQAR
1191.659	1190.651	1190.629	18.2	447 -	456	0	VYEGERPLTK
1210.622	1209.614	1209.578	30.1	359 -	368	1	EFFNGKEPSR
1528.764	1527.756	1527.739	11.3	307 -	318	1	AKFEELNMDLFR 1 Oxidation (M)
1555.802	1554.794	1554.786	5.1	29 -	42	1	NGRVEIIANDQGNR
1566.792	1565.784	1565.773	7.5	43 -	56	0	ITPSYVAFPTPEGER
1801.905	1800.897	1800.889	4.4	541 -	555	1	IDTRNELESYAYSLK
1815.976	1814.968	1814.989	-11.5	180 -	196	1	INEPTAAAIAYGLDKR
1833.890	1832.882	1832.902	-10.6	64 -	79	1	NQLTSNPENTVFDAGR
1887.965	1886.957	1886.964	-3.5	147 -	163	0	VTHAVVTVPAYFNDAQR
1933.993	1932.985	1933.006	-10.6	457 -	474	0	DNHLLGTFDLTGIPPAPR
2016.042	2015.034	2015.059	-12.2	146 -	163	1	KVTHAVVTVPAYFNDAQR



NIMR Customised Mascot Protein View

Search title: 1. . . (click here for default protein view)

Match to gi|6470150 BiP protein [Homo sapiens]

Cleavage by Trypsin: cuts C-term side of KR unless next residue is P

Data file: ../data/20041207/F007367.dat (rank: 1)

Taxonomy: Homo sapiens

BLAST gi|6470150 at NCBI against nr

Matched peptides shown in **Bold Black** and unmatched H, K, R in Red

1 MEEDKKEDVG TVVGIDLGTT YSCVGVFKNG RVEIIANDQG NRITPSYVAF TPEGERLIGD AAKNQLTSNP
71 **ENTVFD**AKRL IGRTWNDPSV QQDIKFLPFK VVEKKIKPYI QVDIGGGQTK TFAPPEISAM VLTMMKETAE
141 **AYLGK**KVTHA **VVTVP**AYFND **AQRQA**TKDAG TIAGLNMVRI **INEPTAAAIA** **YGLDK**REG EK NILVFDLGGG
211 TFDVSLLTID NGVFEVVATN GDTHLGGEDF DQRMVEHFIK LYKKKTGKDV RKDNRAVQKL RREVEKAKRA
281 **LSSQH**QARIE IESFYEGEDF SETLTRAKE **ELNMDL**FRST MKPVQKVED SDLKKSIDE IVLVGGSTRI
351 **PKIQQLVKEF** **FNGKEP**SRGI NPDEAVAYGA AVQAGVLSGD QDTGDLVLLD VCPLTLGIET VGGVMTKLIP
421 RNTVVPTKKS QIFSTASDNQ PTVTIKVYEG **ERPLTK**DNHL **LGTFDLTGIP** **PAPRG**VPQIE VTFEIDVNGI
491 LRVTAEDKGT GNKNKITITN DQNRLTPEEI ERMVNDAEKF AEEDKKLKER **IDTRNE**LESY **AYS**LKNQIGD
561 **KEKLGG**KLSS EDKETMEKAV EEKIEWLESH QDADIEDFKA **KKKELE**EIVQ PIISKLYGSA GPPPTGEEDT
631 AELHHHHHH

Nominal mass of protein (M_r): 71002

Sequence Coverage: 25%

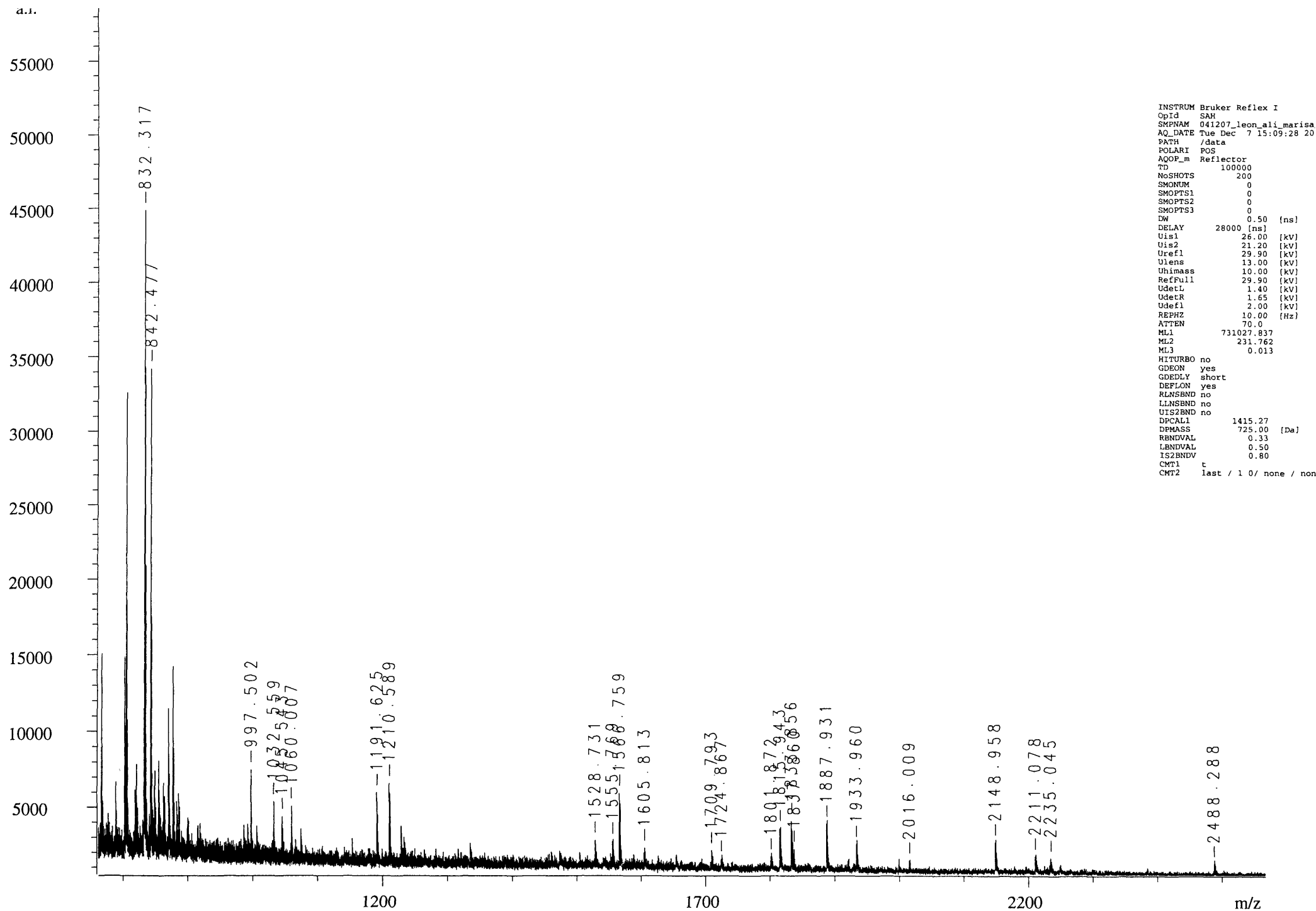
Masses for matched peptides shown in bold black in the following list:

Masses of unobserved peptides ([HR]) containing, completely cleaved & unmodified) are shown in blue

Start - End	Observed	Calc.	± ppm	Miss	Sequence
244 - 250		903.476		0	(..DQR)VMEHFIK(LYK..)
515 - 522		986.516		0	(..QNR)LTPEETER(MVN..)
280 - 288	997.535	997.518	17.2	0	ALSSQH QAR
506 - 514		1074.554		0	(..KNK)ITITNDQNR(LTP..)
447 - 456	1191.659	1191.637	18.2	0	VYEGER PLTK
359 - 368	1210.622	1210.586	30.1	1	EFFNGKEP SR
168 - 179		1217.631		0	(..ATK)DAGTIAGLNMVRI(IIN..)
32 - 42		1228.629		0	(..NCR)VEIIANDQGNR(ITP..)
309 - 318		1313.620		0	(..RAK)FEELNMDLFR(STM..)
336 - 349		1460.760		0	(..LKK)SDIDEIVLVGGSTR(IPK..)
307 - 318		1512.752		1	AKFEELNMDLFR
307 - 318	1528.764	1528.747	11.2	1	AKFEELNMDLFR 1 Oxidation (M)
29 - 42	1555.802	1555.794	5.1	1	NGRVEIIANDQGNR
43 - 56	1566.792	1566.780	7.4	0	ITPSYVAF TPEGER
541 - 555	1801.905	1801.897	4.3	1	IDTRNE LESYAYS LK
180 - 196	1815.976	1815.997	-11.5	1	IINEPTAAAIA YGLDKR
64 - 79	1833.890	1833.909	-10.6	1	NQLTSNP ENTVFD AKR
147 - 163	1887.965	1887.972	-3.5	0	VTHAVV TVPAYFNDAQR
457 - 474	1933.993	1934.014	-10.6	0	DNHLLG TFDLTGIPPAPR
584 - 599		1974.908		0	(..EEK)IEWLESHQDADIEDFK(AKK..)
475 - 492		1999.086		0	(..APR)GVPQIEVTFEIDVNGILR(VTA..)
146 - 163	2016.042	2016.067	-12.2	1	KVTHAVV TVPAYFNDAQR
289 - 306		2164.993		0	(..QAR)IEIESFYEGEDFSETLTR(AKF..)
43 - 63	2235.079	2235.166	-39.0	1	ITPSYVAF TPEGERLIGDAK
616 - 639		2626.182		0	(..ISK)LYGSAGPPPTGEEDTAELHHHHHHH(..)
201 - 243		4567.227		0	(..GEK)NILVFDLGGGTFDVSLLTIDNGVFEVVATNGDTHLGGEDFDQR

Mean error = 13.9 ppm

Sort peptides by: ☐ Residue Number ☒ Increasing Mass ☐ Decreasing MassFTP lists to TOF-MS ☐ No ☒ Yes --- Annotation label: 1Potential phosphorylation? ☒ No ☐ YesPotential anomolous cleavages? ☒ No ☐ YesLysine containing missed peptides? ☒ No ☐ YesAppend entrez report? ☒ No ☐ Yes



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ProtParam tool

User-provided sequence:

1	11	21	31	41	51	
1	MRGSHHHHHH	GSAEKQERVQ	HLHDIRDLHR	YYSSESFEYS	NVSGKVENYN	GSNVVRFNPK 60
61	DQNHQLFLLG	KDKEQYKEGL	QGQNVFVVQE	LIDPNGLRLST	VGGVTKKNNK	TSETNTPLEFV 120
121	NKVNGEDLDA	SIDSFLIQKE	EISLKELDFK	IRQQLVNNYG	LYKGTSKYKG	IIINLKDENK 180
181	VEIDLGDKLQ	FERMGDVLNS	KDIRGISVTI	NQI		

References and documentation are available.

Number of amino acids: 213

Molecular weight: 24439.3

Theoretical pI: 6.80

Amino acid composition:

Ala (A)	2	0.9%
Arg (R)	9	4.2%
Asn (N)	19	8.9%
Asp (D)	14	6.6%
Cys (C)	0	0.0%
Gln (Q)	13	6.1%
Glu (E)	16	7.5%
Gly (G)	17	8.0%
His (H)	10	4.7%
Ile (I)	14	6.6%
Leu (L)	19	8.9%
Lys (K)	20	9.4%
Met (M)	2	0.9%
Phe (F)	8	3.8%
Pro (P)	3	1.4%
Ser (S)	16	7.5%
Thr (T)	7	3.3%
Trp (W)	0	0.0%
Tyr (Y)	8	3.8%
Val (V)	16	7.5%
Asx (B)	0	0.0%
Glx (Z)	0	0.0%
Xaa (X)	0	0.0%

Total number of negatively charged residues (Asp + Glu): 30

Total number of positively charged residues (Arg + Lys): 29

Atomic composition:

Carbon C 1074

Hydrogen	H	1700
Nitrogen	N	312
Oxygen	O	337
Sulfur	S	2

Formula: $C_{1074}H_{1700}N_{312}O_{337}S_2$

Total number of atoms: 3425

Extinction coefficients:

Conditions: 6.0 M guanidium hydrochloride
0.02 M phosphate buffer
pH 6.5

Extinction coefficients are in units of $M^{-1} \text{ cm}^{-1}$.

	276	278	279	280	282
	nm	nm	nm	nm	nm
Ext. coefficient	11600	11200	10760	10240	9600
Abs 0.1% (=1 g/l)	0.475	0.458	0.440	0.419	0.393

Estimated half-life:

The N-terminal of the sequence considered is M (Met).

The estimated half-life is: 30 hours (mammalian reticulocytes, in vitro).
>20 hours (yeast, in vivo).
>10 hours (Escherichia coli, in vivo).

Instability index:

The instability index (II) is computed to be 15.71
This classifies the protein as stable.

Aliphatic index: 83.15

Grand average of hydropathicity (GRAVY): -0.822

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SETS

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ProtParam tool

User-provided sequence:

```

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      |      |      |      |      |      |
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61 NFNQRNKNFK VFLIGDDRNK YKELTHGRDV FAVPELIDTK GGIYSVGGIT KKNVRSVFGY    120
121 VSHPGQLQVKK VDPKDGFSIK ELFFIQKEEV SLKELDFKIR KMLVEKYRLY KGASDKGRIV    180
181 INMKDEKKHE IDLSEKLSFD RMFDVLDSKQ IKNIEVNLN
  
```

[References and documentation](#) are available.

Number of amino acids: 219

Molecular weight: 25606.1

Theoretical pI: 9.31

Amino acid composition:

Ala (A)	3	1.4%
Arg (R)	10	4.6%
Asn (N)	14	6.4%
Asp (D)	16	7.3%
Cys (C)	0	0.0%
Gln (Q)	7	3.2%
Glu (E)	15	6.8%
Gly (G)	14	6.4%
His (H)	9	4.1%
Ile (I)	14	6.4%
Leu (L)	16	7.3%
Lys (K)	29	13.2%
Met (M)	5	2.3%
Phe (F)	11	5.0%
Pro (P)	3	1.4%
Ser (S)	17	7.8%
Thr (T)	6	2.7%
Trp (W)	0	0.0%
Tyr (Y)	12	5.5%
Val (V)	18	8.2%
Asx (B)	0	0.0%
Glx (Z)	0	0.0%
Xaa (X)	0	0.0%

Total number of negatively charged residues (Asp + Glu): 31

Total number of positively charged residues (Arg + Lys): 39

Atomic composition:

Carbon	C	1147
Hydrogen	H	1807

Nitrogen N 317
Oxygen O 338
Sulfur S 5

Formula: $C_{1147}H_{1807}N_{317}O_{338}S_5$

Total number of atoms: 3614

Extinction coefficients:

Conditions: 6.0 M guanidium hydrochloride
0.02 M phosphate buffer
pH 6.5

Extinction coefficients are in units of $M^{-1} cm^{-1}$.

	276	278	279	280	282
	nm	nm	nm	nm	nm
Ext. coefficient	17400	16800	16140	15360	14400
Abs 0.1% (=1 g/l)	0.680	0.656	0.630	0.600	0.562

Estimated half-life:

The N-terminal of the sequence considered is M (Met).

The estimated half-life is: 30 hours (mammalian reticulocytes, in vitro).
>20 hours (yeast, in vivo).
>10 hours (Escherichia coli, in vivo).

Instability index:

The instability index (II) is computed to be 22.90
This classifies the protein as stable.

Aliphatic index: 78.63

Grand average of hydropathicity (GRAVY): -0.765

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Structural Relationships and Cellular Tropism of Staphylococcal Superantigen-Like Proteins

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